AMP and its role in asthma

M. van den Berge

AMP and its role in asthma

Maarten van den Berge

2003

van den Berge, M.

AMP and its role in asthma.

Thesis University Hospital Groningen with summary in Dutch

ISBN: 9036719089

Cover design by Mayda Stoel.
Printed by Drukkerij Ponsen en Looijen BV.

The printing of this thesis was financially supported by:

Main sponsor: GlaxoSmithKline BV

Altana Pharma, Astra Zeneca, MSD, Boehringer Ingelheim, Aventis, Pfizer, Jaeger Toennies Benelux BV, Guide, ABBOTT BV., Stichting astmabestrijding, Amgen, Schering-Plough, UCB Pharma, 3M, Novartis, Zambon, Christiaens BV.

Stellingen behorend bij het proefschrift

AMP and its role in asthma

Maarten van den Berge, 26 november 2003

1. Een lage PC_{20} methacholine is geen garantie voor uitgebreide inflammatie in de luchtwegen.

2. Een meting van PC_{20} AMP geeft een goed inzicht in de actuele staat van inflammatie in de luchtwegen.

3. Verandering in luchtweginflammatie na behandeling met corticosteroïden wordt beter "opgepikt" met een PC_{20} AMP dan met een PC_{20} methacholine meting.

4. Een provcatietest met AMP leidt, in tegenstelling tot een provocatietest met methacholine, tot een toename van het aantal eosinofiele granulocyten in het sputum.

5. Inhalatie van een adenosine A_{2A}-agonist (GW328267X), 25 µg twee keer per dag, is niet geschikt als behandeling voor astma.

6. De toenemende regelgeving bij wetenschappelijk onderzoek werkt soms adembenemend.

7. Een proefschrift is net als een pil. Het is even slikken, maar je wordt er wel beter van.

8. Creatief omgaan met sponsorgelden binnen de gestelde kaders is geen belastingontduiking.

Rijksuniversiteit Groningen

AMP and its role in asthma

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr F. Zwarts,
in het openbaar te verdedigen op
woensdag 26 november 2003
om 16.00 uur

door

Maarten van den Berge

geboren op 28 februari 1974
te Deventer

Promotores: Prof. dr. D.S. Postma
Prof. dr. G.H. Koëter
Prof. dr. H.A.M. Kerstjens

ISBN: 9036719089

Beoordelingscommissie:	Prof. dr. S.T. Holgate
	Prof. dr. J.W.J. Lammers
	Prof. dr. P.J. Sterk
	Prof. dr. J.G.R. de Monchy
Paranimfen:	M.A.J.L. Lavèn
	Drs. L. van den Berge

Contents

Chapter 1	General Introduction	7
Chapter 2	Provocation with Adenosine 5'-Monophosphate as a marker of inflammation in asthma, allergic rhinitis and chronic obstructive pulmonary disease *Clin Exp Allergy 2002; 32:824-830*	37
Chapter 3	PC_{20} AMP is more closely associated with airway inflammation in asthma than PC_{20} methacholine *Am J Respir Crit Care Med 2001; 163:1546-1550*	55
Chapter 4	Corticosteroid-induced improvement in the PC_{20} of adenosine monophosphate is more closely associated with reduction in airway inflammation than improvement in the PC_{20} of methacholine *Am J Respir Crit Care Med 2001; 164:1127-1132*	71
Chapter 5	Provocation with AMP, but not methacholine, induces sputum eosinophilia	87
Chapter 6	Effect of an inhaled adenosine A_{2A}-agonist on the allergen induced late asthmatic response. A double-blind, placebo- and fluticasone controlled study	101
Chapter 7	Altered β_2-adrenergic regulation of T cell activity after allergen challenge in asthma	117
Chapter 8	Summary, conclusions, discussion and directions for future research	135
Chapter 9	Samenvatting in het Nederlands	151
Chapter 10	Dankwoord	159

Chapter 1

General Introduction

Asthma is now accepted as a disease characterized by airway inflammation and recurrent episodes of symptoms of wheezing and chest tightness that are associated with variable airway obstruction and increased bronchial hyperresponsiveness.[1] In addition, accumulating evidence is available that airway structural changes or 'remodeling' occur in asthmatic airways, secondary to this inflammatory process.[2] Airway remodeling has in turn been linked to physiological dysfunction, leading to the development of fixed airflow limitation, which can be measured in a subset of asthmatic patients.[3,4] The concept that airway inflammation leads to airway remodeling, thereby possibly worsening the long-term outcome of asthma, has led to the view that the major goal of asthma treatment is improve airway inflammation as much as possible with anti-inflammatory therapy. However, in the present guidelines, the level and adjustment of anti-inflammatory treatment for asthma is guided solely by symptoms and lung function.[5,6] This may not be appropriate, since it has been shown that airway inflammation and bronchial hyperresponsiveness persist in many patients whose disease is considered to be clinically controlled.[7,8] In addition, it has been shown that the direct association between symptoms on the one hand and airway inflammation on the other hand is either only weak or absent.[7] Thus, it has been suggested that asthma management can be improved by incorporating an objective marker of inflammation.[9] For this reason, there has been an intensive search in the last decade to find objective markers of airway inflammation in asthma, which are non-invasive and can be used easily. It has been suggested that the severity of bronchial hyperresponsiveness is such a measure.[9]

AIRWAY INFLAMMATION IN ASTHMA

The nature of the inflammatory reaction in asthma has been the subject of intensive investigation by advanced techniques in peripheral blood, induced sputum, bronchial biopsies, and brochoalveolar lavage specimens and exhaled breath. Airway inflammation has thus been found not only in those with far advanced disease or dying from intractable asthma, but also in newly diagnosed asthmatics with clinically mild disease.[10-13] The asthmatic inflammatory process is characterized by a complex interplay of resident cells (i.e. epithelial and dendritic cells, fibroblasts, nerves, endothelial cells) and inflammatory cells (eosinophils, mast cells, neutrophils, macrophages, T-lymphocytes).[14] Communication is based on cell-cell interactions (ICAM)-1, VCAM-1), cytokines (IL-3, IL-4, IL-5, GM-CSF, TNF-alpha, IL-1-beta) and other cell derived mediators (prostaglandins, leukotrienes, histamine, RANTES, eotaxin). Inhaled allergen challenge may be used as a model to understand inflammation in asthma. An allergen challenge leads to an early allergic inflammatory reaction in allergic asthmatic patients and in approximately 70% of cases this is followed by a late asthmatic reaction.[15]

Early asthmatic reaction

The early asthmatic reaction occurs after the activation of mast cells bearing the specific high affinity IgE receptor FcεR1.[16-19] Cell types other than mast cells that bear the FcεR1 receptor may also participate, but it is not known whether they can be activated directly by allergens.[20,21] The activated cells rapidly release pro-inflammatory mediators such as histamine and leukotrienes which induce contraction of airway smooth muscle, mucous secretion, and microvascular leakage.[22,23]

Late asthmatic reaction

The late asthmatic reaction occurs between 2 and 9 hours after allergen challenge and involves the recruitment and activation of peripheral blood cells, including eosinophils, $CD4^+$ T cells, basophils, neutrophils, and macrophages.[24-27] The recruitment of these cells is the result of interaction between circulating inflammatory cells and resident cells (especially endothelial cells) and is characterized by the complex interplay of proinflammatory mediators, cytokines, and chemokines, and cell surface adhesion molecules.[28,29] Together, the effects occuring during the early and late asthmatic reaction contribute to an increase in airway obstruction and bronchial hyperresponsiveness.

AIRWAY REMODELING IN ASTHMA

Morphometric studies have revealed that the airway wall of asthma patients is characterized by an increased thickness involving goblet cell hyperplasia, a thickened area below the basement membrane, smooth muscle hyperplasia and hypertrophy, mucous gland hyperplasia, and increased vascularity.[12,30-32] Together, these changes in asthmatic airways are commonly referred to as 'airway remodeling'.[2] Interestingly, recent studies have shown an association between a thicker area below the basement membrane (one of the key components of airway remodeling) and both a lower forced expiratory volume in one second (FEV_1) and more severe bronchial hyperresponsiveness.[33-36] In addition, it has been shown in a recent study of Benayoun and colleagues that a thicker area below the basement membrane, a larger mucous gland mass, and an increase in airway smooth muscle area and airway smooth muscle cell size are associated with more severe asthma.[37] Thus, it appears that airway remodeling contributes to airway obstruction and bronchial hyperresponsiveness in asthma and it is tempting to speculate that airway remodeling is of critical importance for disease progression in asthma.

Although medical textbooks generally report asthma to be a benign disease, without an evident lung function loss over time, this appears to be a too simple statement, since various degrees of airway inflammation may persist in asthma and, in the long term, asthma may become moderately to fully irreversible in a subset of patients.[3,4] It was found by Vonk and colleagues that approximately 15% of asthmatic patients will develop some degree of irreversible airway obstruction over a period of 30 years.[38] Generally, lung function increases during childhood; it reaches a plateau during early adulthood (18-35 years) and declines after the age of 35 years.[39] There is a paucity of data relating to the effect of childhood asthma on the growth of lung function, but it has been demonstrated in a few studies that more severe asthma in childhood is associated with a lower level of lung function in adulthood.[40-43] In a population based study of Weiss and colleagues children were followed for over 13 years.[44] It was found in that study that the presence of persistent active asthma has a progressively negative impact on the annual change in FEV_1 in young females, but not in young males. Based on their analyses, the authors predicted that a female, who develops asthma at 7 years of age will experience a 5% reduction in FEV_1 by 10 years of age and a 7% deficit by 15 years of age. These observations suggest that gender has a negative impact on the annual change in FEV_1 in childhood, although this might have been confounded by the severity of the disease as female asthmatics were hospitalized more frequently for asthma than male asthmatics. Taken together, it seems reasonable to

conclude that asthmatic children are at risk to have persistently lower lung function levels than their healthy counterparts when they reach adulthood. Some longitudinal studies have now provided evidence that a subgroup of adult asthmatics may have an increased decline in lung function during their adult life as well.[45-47] The magnitude of the excess decline in lung function in asthmatics above the normal decline of 20-30 ml, however, differs between the studies ranging from 5 to 25 ml/year.[40,46] In addition, Ulrik and colleagues have demonstrated in never-smoking asthmatics that there exists a subset of asthmatics with an excess decline in lung function ultimately leading to a severe, non-reversible airway obstruction without the presence of emphysema.[48] In this study, the decline in lung function was highest in patients who initially had very variable airflow limitation, which suggests that an excess decline in lung function reflects the long-term consequences of poorly controlled airway inflammation. In agreement with this, several cross-sectional studies have shown that a lower lung function is associated with a longer duration of asthma and a longer delay from onset of respiratory symptoms until referral for specialist treatment.[49,50] Furthermore, in a longitudinal study, Grol and colleagues found that subjects with asthma who continued to use inhaled corticosteroids had a less steep decline in lung function over a period of 10 years than those who had stopped their corticosteroid treatment.[51] Taken together, there is now circumstantial evidence that a higher annual decline in FEV_1 may occur in a subset of asthmatics. It is well conceivable that this is due to the persistence of airway inflammation, which leads to the development of progressive airway remodeling. This may provide an explanation for the fixed airway obstruction, which can be observed in a subset of asthmatic patients.[3,4,38] Thus, epidemiological data would suggest that a major goal of asthma management is to suppress airway inflammation as much as possible for instance with inhaled corticosteroids, as has also been suggested by many guidelines.[5,6,52]

RELATIONSHIP BETWEEN SYMPTOMS, LUNG FUNCTION, AND AIRWAY INFLAMMATION IN ASTHMA

According to present guidelines, the treatment of asthma is solely based on symptoms and lung function.[5,6,52] However, it has been shown in several studies that the direct association between symptoms on the one hand and lung function and airway inflammation on the other hand is either only weak or absent and therefore this may not be appropriate.[7] In addition, it has been demonstrated that airway inflammation may persist in many patients whose asthma is considered to be clinically controlled.[8,9,53-55] This is in agreement with the findings of de Kluijver and colleagues who demonstrated that repeated low dose allergen exposure is accompanied by an increase in airway inflammation, as reflected by the percentage of sputum eosinophils, without a concomitant worsening of asthma symptoms.[56] Further evidence for a poor association between airway inflammation and symptoms in asthma can be derived from a recent study by van den Toorn and colleagues in subjects in 'clinical remission' of their asthma. In this study, asthmatics were regarded in clinical remission if they had a documented history of asthma, but reported a complete absence of cough, chest discomfort, or breathlessness for at least one year preceding the study and did not use any medication in order to control asthma.[57,58] Interestingly, these subjects exhibited a higher level of airway inflammation and bronchial hyperresponsiveness to methacholine and AMP than nonasthmatic control subjects. In addition, they had a thicker reticular area below the basement membrane, indicating the presence of airway remodeling. It has been shown that a considerable percentage of patients who have outgrown their asthma will have a relapse later in life.[59] Thus, it is tempting to speculate that the persistence of airway

inflammation together with progressive airway remodeling may ultimately lead to a relapse of asthma symptoms in a subset of subjects who are 'in clinical remission'. It can be speculated from the above that asthmatic subjects or subjects who have outgrown their asthma may benefit from anti-inflammatory therapy with inhaled corticosteroids, independent of their level of lung function or the presence of asthmatic symptoms. However, this has to be confirmed in future studies.

MARKERS OF AIRWAY INFLAMMATION IN ASTHMA

The cellular and immunologic markers of airway inflammation can be studied by sampling procedures of the airway (bronchoalveolar lavage, bronchial biopsies, bronchial brushings, and induced sputum), or in peripheral blood.[60] In addition, a number of (by-)products of airway inflammation can be found in exhaled air.[61] Thus, the analysis of constituents of exhaled air provides a potentially easily accessible, non-invasive method of monitoring inflammation. Finally, it has been suggested that measurement of bronchial hyperresponsiveness may be useful as a surrogate marker to monitor airway inflammation in asthma.[9]

Bronchial biopsies and bronchoalveolar lavage fluid

The current gold standard by which to determine airway inflammation remains bronchial biopsy.[62-64] Bronchial biopsies offer several advances: 1) they directly reflect pathologic changes occurring in the airway wall mucosa and submucosal region, 2) they can be examined by many methods including light and electron microscopy, 3) the state of activation of cells and their secretory products can be assessed, and 4) the localization of cells within the submucosa and epithelium can be analyzed.[65,66] One further can use bronchoalveolar lavage, which samples the more peripheral airways and allows more quantitative measures of mediators released in the airways. Studies with bronchial biopsies and bronchoalveolar lavage fluid have revealed an increased number and activation state of inflammatory cells such as eosinophils, lymphocytes, and mast cells accompanied by their respective mediators when compared with subjects without asthma.[67-69] In addition, an increased asthma severity has been associated with increased numbers and activation of airway eosinophils.[70] Furthermore, macrophage activation in bronchoalveolar lavage fluid has been described in symptomatic asthma and in asthmatics with nocturnal exacerbations.[19,71] While neutrophilia in bronchial biopsies or bronchoalveolar lavage fluid is not a predominant feature of mild asthma, it has been demonstrated in more severe asthma.[72] Many cytokines and mediators have been detected in bronchoalveolar lavage fluid including TNF-α, GM-CSF, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-13, soluble adhesion molecules, leukotrienes, histamine, and tryptase.[67] A major disadvantage of both bronchial biopsies and bronchoalveolar lavage fluid is that these procedures are invasive and therefore unsuitable for repeated or routine use. For this reason, there has been extensive research over the past decade to find a suitable non-invasive marker of airway inflammation in asthma.

Sputum

The use of induced sputum has been proposed as an alternative method to measure airway inflammation in asthma.[73,74] Hypertonic saline is used to elicit sputum production when

sputum could not be expectorated spontaneously.[73] Once the sputum has been dissolved, a cell differential count can be performed after staining. In addition, mediators such as eosinophil cationic protein (ECP), histamine, tryptase, markers of mucus secretion, and neutrophil-derived markers can be measured in the sputum supernatant.[75-78] Significant correlations have been observed in asthma patients between the number of eosinophils in sputum on the one hand and the number of eosinophils in bronchoalveolar lavage fluid and biopsies on the other hand.[79] Further, asthmatic subjects have higher numbers of inflammatory cells such as eosinophils and metachromatic cells in induced sputum when compared to nonasthmatic subjects.[80-82] Sputum induction is also responsive to changes in asthma control, with increases in the number of eosinophils and neutrophils during asthma exacerbations or in the number of eosinophils after allergen inhalation.[83,84] Thus, examination of induced sputum is considered to represent a useful tool for assessment of the degree of airway inflammation in asthma. Despite this positive notion, sputum induction is unlikely to have a place in asthma management in the foreseeable future given the drawbacks that limit its use in daily clinical practice. Dedicated, highly qualified laboratory technicians are needed and the technique is time consuming, preventing rapid help in decision making to change treatment. In addition, the sputum induction procedure takes up to one hour and processing of the sputum to the necessary provision of percentage eosinophils approximately three hours.

Peripheral blood

Cell counts and measurement of inflammatory mediators in peripheral blood may provide a reflection of the degree of airway inflammation in asthma. The number of blood eosinophils and the concentration of eosinophil cationic protein (ECP) is increased in asthma when compared to control samples and decreases after therapy with inhaled corticosteroids.[85,86] A significant correlation has been found between the number of eosinophils in bronchial biopsies and the concentration of ECP in blood, although the correlation is only weak.[87] In most studies, however, there is a large scatter in the data for peripheral blood eosinophils and ECP concentrations and there are several studies in which no decrease in the number of eosinophils and the concentration of serum ECP could be found after therapy with inhaled corticosteroids.[60,88] For this reason, it has been suggested by some authors that assessment of cell counts and mediators in peripheral blood is not useful as it fails to reflect disease activity in sufficient detail.[89-92]

Exhaled nitric oxide

Endogenous nitric oxide (NO) is derived from L-arginine by the enzyme nitric oxide synthase. The increase in exhaled NO in asthma patients is mainly caused by induction of nitric oxide synthase by proinflammatory cytokines.[61] Exhaled NO can be analyzed by chemiluminescence and is expressed as a concentration in parts per billion.[61] Exhaled NO, which can be measured very easily, has been put forward as a new way to monitor airway inflammation in asthma. Increased levels of exhaled NO have been widely documented in patients with asthma when compared to healthy control subjects.[93-95] In addition, the level of exhaled NO increases further after allergen exposure and decreases after anti-inflammatory therapy with corticosteroids.[96-98] Furthermore, it was found that exhaled NO is as useful as sputum eosinophils and bronchial hyperresponsiveness to hypertonic saline for predicting loss of asthma control after withdrawal of inhaled corticosteroids.[99] However,

there also drawbacks to the use of exhaled NO. Exhaled NO is extremely sensitive to steroid treatment as it is significantly reduced already 6 hours after a single dose of a nebulized corticosteroid.[100] In a recent study, treatment with a low dose of inhaled corticosteroids significantly reduced the level of exhaled NO, but not the percentage of sputum eosinophils in subjects with asthma.[101] This may suggest that exhaled NO is too sensitive to steroids per se to determine whether airway inflammation is adequately controlled and therefore exhaled NO may be less useful as tool to monitor effects of anti-inflammatory treatment with inhaled corticosteroids in asthma.

Bronchial hyperresponsiveness

Although airway inflammation and bronchial hyperresponsiveness are recognized as major characteristics of asthma, their relationship is still poorly understood. It has been suggested in several studies that bronchial hyperresponsiveness is closely associated with airway inflammation, since both the severity of bronchial hyperresponsiveness and the percentage of sputum eosinophils increase after allergen exposure.[102,103] Furthermore, bronchial hyperresponsiveness and sputum eosinophilia improve after therapy with inhaled corticosteroids.[88] An association between bronchial hyperresponsiveness and airway inflammation might be expected, since activation of different inflammatory cells leads to airway wall edema with a concomitant increase in airway wall thickness. In addition, smooth muscle cells become more sensitive to contracting stimuli.[104] This all contributes to an increase in bronchial responsiveness. However, other factors also contribute to the presence and severity of bronchial hyperresponsiveness. Recent morphologic and functional studies have demonstrated that bronchial hyperresponsiveness may be compounded by airway remodeling.[105] It can be inferred from the above that the presence of a direct association between bronchial hyperresponsiveness and airway inflammation remains questionable. Indeed, there have been almost as many negative as positive reports in the literature.[7,106-109] Thus far, bronchial hyperresponsiveness has usually been measured with methacholine or histamine. These are both direct stimuli, since they exert their effect directly on airway smooth muscle. Another possible stimulus to measure bronchial hyperresponsiveness is Adenosine 5' Monophosphate (AMP). AMP is an indirect stimulus, since it has little effect on airway smooth muscle contraction 'in vitro'.[110] A major action of AMP appears to involve the release of histamine and other preformed mediators from immunologically primed mast cells, since AMP-induced bronchoconstriction is associated with a rise of histamine in plasma and bronchoalveolar lavage fluid.[111,112] Moreover, the concentration of AMP causing the FEV_1 to drop by 20% (PC_{20}) is inhibited up to 80% by pretreatment with antihistamines in asthma.[113,114] Interestingly, it has been suggested that the severity of PC_{20} AMP is more closely associated with airway inflammation than the severity of PC_{20} methacholine, since it has been shown that the PC_{20} AMP improves after a stay of one month in a hypoallergenic environment at high altitude, whereas PC_{20} methacholine remains stable (Switzerland, Davos) (figure 1).[115] In addition, the PC_{20} AMP improves to a larger extent after therapy with corticosteroids than PC_{20} methacholine.[88,116-118] Thus, the PC_{20} AMP may be a new promising non-invasive marker of airway inflammation in asthma and therefore its exact value needs to be investigated further in future studies.

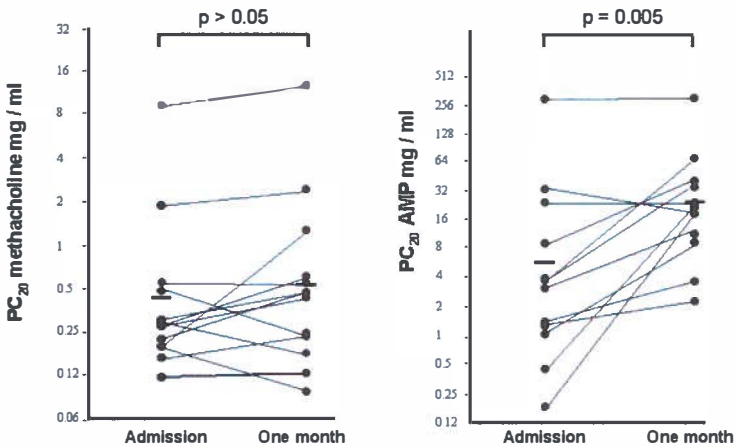


Figure 1. Changes in PC_{20} methacholine and in PC_{20} AMP after a one month stay in a hypoallergenic environment at high altitude in Switzerland, Davos *(reprinted with permission from reference 115).*

MECHANISM OF ACTION OF AMP

Once inhaled, AMP is rapidly broken down to adenosine by the ubiquitous enzyme 5'-nucleotidase. Adenosine exerts its effect via extracellular cell surface receptors.[119,120] Thus far, four different adenosine receptors have been described, namely adenosine A_1, A_{2A}, A_{2B}, and A_3 receptors.[121] Human lung mast cells have been shown to express A_{2A} and A_{2B} receptors, but not A_1 or A_3 receptors.[122,123] In a recent study, only activation of the A_{2B} receptor resulted in mast cell activation.[124] Thus, there is suggestive evidence that AMP-induced bronchoconstriction is mediated by activation of the A_{2B} receptor.

Adenosine can also act on A_1, A_{2A}, and A_3 receptors, which have been identified on neutrophils, eosinophils, and macrophages. Their activation has various pro- and anti-inflammatory actions: activation of adenosine A_1 receptors promotes chemotaxis of neutrophils and increases adherence of neutrophils to endothelial cells.[125-127] By contrast, activation of adenosine A_{2A} receptors reduces chemotaxis, activation and degranulation of neutrophils.[126,128,129] In addition, activation of A_{2A} receptors (in contrast to activation of A_{2B} receptors) suppresses the release of tryptase from mast cells.[130] A_3 receptors mediate inhibition of eosinophil chemotaxis when activated.[122] Other functions of A_3 receptors involve inhibition of neutrophil degranulation induced by LPS or TNF-alpha.

ROLE OF ADENOSINE IN ASTHMATIC AIRWAY INFLAMMATION

Adenosine is a naturally occurring purine nucleoside with a ubiquitous presence in human tissue. AMP is formed by the catabolism of high-energy adenosine phosphates such as adenosine diphosphate (ADP) or adenosine triphosphate (ATP) during the process of energy generation. When sufficient oxygen and energy is available, AMP is reconverted to high-energy adenosine phosphates forming a component of the energy cycle.[131] However, in case of excessive cell stimulation or hypoxia, AMP can not be reconverted and is

transported to the exterior of the cell where it is metabolized to adenosine by the enzyme 5'-nucleotidase. The discovery that adenosine levels are elevated in the bronchoalveolar lavage fluid (figure 2) and in exhaled breath of asthmatics and increase further after allergen challenge raises the possibility that adenosine, once generated in asthmatic airways, itself contributes to the pathogenesis of asthma.[132-134] An explanation of the observed increase in adenosine could be that stimulation of inflammatory cells and subsequent smooth muscle contraction during the asthmatic inflammatory process leads to an increase in oxygen and energy demand, thus inducing an increase in the level of adenosine.

Animal models provide an opportunity to specifically investigate the role of adenosine in asthma. It has thus been shown that mice which lack the enzyme adenosine deaminase (and are therefore unable to break down endogenously formed adenosine) develop features of asthma including bronchial hyperresponsiveness, enhanced mucus secretion, airway eosinophilia, and increased IgE synthesis.[135,136] These asthmatic features were reversed by exogenous administration of adenosine deaminase. Transcript array technology was used to examine which genes in the lung become activated by adenosine when accumulated in adenosine deaminase-deficient mice. Thus, overexpression of the monocyte chemotactic protein-3 (MCP-3) gene in the airway was found and paralleled by enhanced MCP-3 secretion. MCP-3 is a chemokine with potent eosinophil chemotactic properties. The same study showed greatly enhanced airway expression of molecules involved in tissue remodeling, including vascular endothelial growth factor, osteopontin, and fibronectin, as well as the cathepsin family of neutral proteases. This provides a further important link between adenosine and asthma. Thus, taken together with the presence of elevated levels of adenosine in the airways, it is now clear that the exact role of adenosine and its receptors in asthma becomes of increasing interest and deserves further study.

One might even consider a therapeutic potential of adenosine receptor antagonists and agonists, since it has been shown in animal models that adenosine A_1 inhibitors and adenosine A_{2A} agonists provide a protective effect on the late asthmatic response after allergen challenge.[137,138] In this context it is of interest that treatment with theophylline, which is also an adenosine A_{2B} antagonist, has been shown to reduce airway inflammation both in asthma and in COPD.[139,140] Although it was originally hypothesized that theophylline acts via inhibition of phosphodiesterases, it has been observed that the concentration of theophylline required to effectively inhibit phosphodiesterases was much greater than the therapeutic level achieved in humans.[141] Since theophylline also inhibits adenosine A_{2B} receptors at concentrations likely to occur 'in vivo', this could well be an alternative explanation for its beneficial effects in asthma.[142-144]

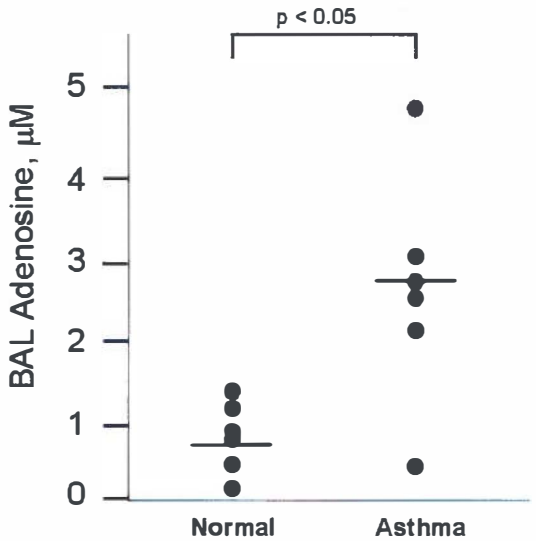


Figure 2. Adenosine concentrations in bronchoalveolar lavage (BAL) fluid *(reprinted with permission from reference 132).*

MAST CELLS

It is unclear whether the increase in the number and activation state of mast cells, which has consistently been reported in asthma, is a feature of allergy or of the asthmatic inflammatory process itself, since allergen exposure can increase the number and activation state of airway mast cells.[145-147] The finding that non-allergic patients with asthma also have a bronchoconstrictor response to AMP suggests that mast cell activation can also be a feature of the asthmatic inflammatory process rather than being dependent on the presence of allergy alone.[113,148] Indeed, both the number and activation state of mast cells were found to be increased in non-allergic patients with asthma and therefore mast cell activation and proliferation can occur under different circumstances than allergen exposure too (figure 3).[149-151] GM-CSF, IL-4, IL-5, IL-9, IL-10, nerve growth factor, and stem cell factor have been demonstrated 'in vitro' to constitute growth factors for human mast cells.[152-155] An increased local production of one or more of these growth factors could be responsible for mast cell accumulation and activation in the airway mucosa. Nevertheless, the number of mast cells is higher in allergic than in non-allergic asthma, indicating an additive effect of allergy on mast cell counts (figure 3).[149] In agreement with this, inhaled adenosine was found to be three times more potent to induce bronchoconstriction in patients with allergic asthma than in patients with non-allergic asthma, whereas another study has shown that the degree of bronchial hyperresponsiveness to a direct stimulus does not differ between both groups.[156,157]

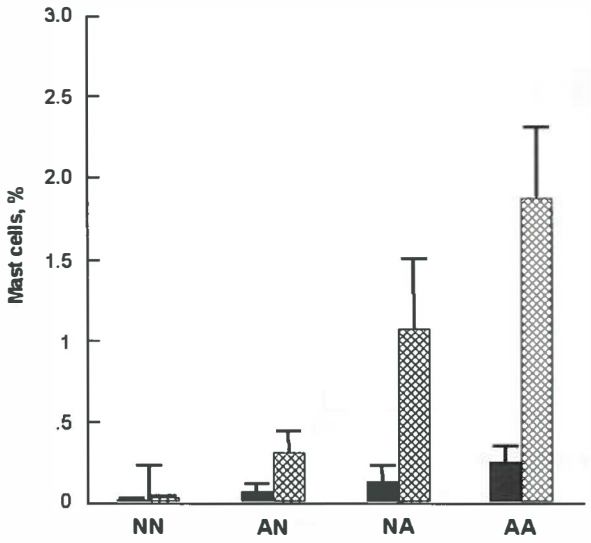


Figure 3. Comparison of mast cell counts in BAL (solid bars) and bronchial brushings (hatched bars) between non-allergic normal (NN), allergic normal (AN), non-allergic asthmatic (NA), and allergic asthmatic (AA) subjects *(reprinted with permission from reference 149).*

In recent years, there has been a tendency to attach less importance to the role of the mast cell as an effector cell in asthma. However, new findings have led to a resurgence of interest in the mast cell and support a reevaluation of its role. In particular, the discovery that mast cells are a source of cytokines has suggested new ways in which mast cell activation could also participate in more persistent and even chronic inflammatory responses.[158-160] The recognition that mast cells produce IL-4 and IL-13 implies that they have the potential to influence IgE production by B cells (figure 4).[161] Further, it has been demonstrated in a study of Gauchat and colleagues that mast cells express CD40 ligand, which enables them to provide the cell-cell signal required for IgE production through the interaction of the ligand with CD40 on B cells.[162] More recently, Pawankar and colleagues reported that mast cells purified from nasal biopsies of dust-mite sensitive patients with allergic rhinitis induce the secretion of IgE from tonsillar B cells when incubated with allergen (figure 4).[163] This mast cell-dependent IgE secretion was partially inhibited by neutralizing monoclonal antibody to IL-4, and completely inhibited by neutralizing antibody to IL-13 and to CD40 ligand (figure 4). It has been demonstrated in several studies that the ability to produce IL-4 and IL-13 enables mast cells to stimulate IgE secretion by B cells.[161-163] This may explain findings of incraesed IgE levels in patients with intrinsic asthma than in non-allergic subjects without asthma, even though the concentration of serum total IgE falls is still lower than in patients with allergic asthma.[164,165] Indeed, it has been found that the observed increased IL-4 levels in non-allergic asthma can be largely attributed to the production by mast cells.[165-167]

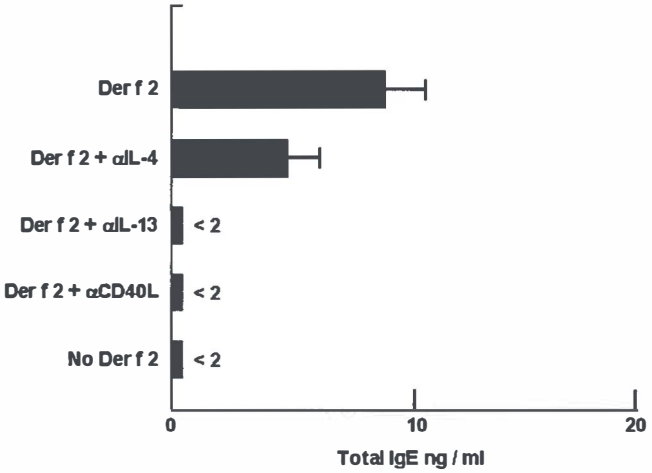


Figure 4. Immunoglobulin E (IgE) synthesis by tonsillar B cells in coculture with nasal mast cells purified from the turbinates of patients with perennial allergic rhinitis. House-dust mite antigen (Der f2)-induced mast cell activation results in B-cell IgE synthesis, an effect that is attenuated by a neutralizing anti-interleukin 4 (IL-4) antibody, and completely abrogated by neutralizing anti-IL-13 and anti-CD40 ligand antibodies (*reprinted with permission from reference 163).*

Further support for an important role of mast cells in non-allergic asthma as well can be derived from a recent animal study of Kraneveld and colleagues.[168] In a mouse model of non-allergic asthma, they induced pulmonary hypersensitivity by cutaneous sensitization followed by intra-airway application of a low molecular weight hapten. The features observed in this mouse model resemble those found in non-allergic asthma and include hapten-induced acute bronchoconstriction, pulmonary edema, infiltration of neutrophils and mononuclear cells, and bronchial hyperresponsiveness, which are not associated with an increase in hapten specific IgE. Interestingly, pulmonary hypersensitivity could not be induced in mast cell deficient mice, unless bone marrow derived mast cells were reconstituted. Taken together, these findings suggest an important role for mast cells in non-allergic and allergic asthma.

MAST CELLS, β_2-RECEPTOR AGONISTS, AND BRONCHOPROTECTION AGAINST THE PC_{20} AMP

It has been demonstrated that the inhaled β_2-receptor agonists albuterol, terbutaline, and formoterol provide greater bronchoprotection against AMP-induced bronchoconstriction than against methacholine or histamine-induced bronchoconstriction (figure 5).[169,170] This differential bronchoprotective effect has been interpreted as mast cell stabilization. In agreement with this, it has been demonstrated that β_2-receptor agonists inhibit the release of histamine from chopped human lung and dispersed human lung mast cells.[171,172]

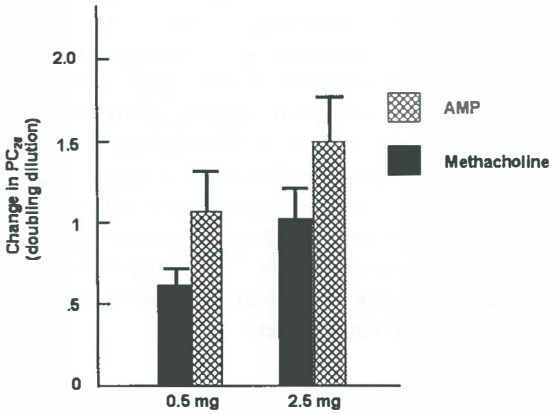


Figure 5. Effect of a single inhalation of terbutaline, 0.5 and 2.5 mg, on bronchoconstrictor responses to methacholine and AMP, expressed a change in PC_{20} in terms of doubling doses (mean ± SEM) compared with placebo *(reprinted with permission from reference 169).*

Furthermore, inhaled albuterol has been shown to inhibit the early increase in plasma histamine induced by allergen exposure in asthmatic patients, although it does not inhibit the late allergen-induced drop in FEV_1.[173]

β_2-receptor agonists also exert an effect on other inflammatory cells. Thus, inhibitory effects on the oxidative burst and the release of thromboxane and leukotriene C4 from eosinophils have been described.[174-176] In addition, β_2-receptors have been detected on circulating neutrophils and β_2-receptor agonists have an inhibitory effect on their mediator release, although relatively high concentrations are required.[177,178] Furthermore, it has recently been shown that β_2-receptor agonists inhibit the release of Th2 type cytokines such as IL-5 from peripheral blood lymphocytes and the release of GM-CSF from cultured human lung fibroblasts.[179,180] Finally, β_2-receptor agonists seem to have an effect on plasma exudation, probably by preventing separation of endothelial cells in postcapillary venules and this effect has also been demonstrated 'in vivo' with terbutaline, formoterol and salmeterol.[181-184]

There have been many studies investigating the effects of β_2-receptor agonists 'in vivo' and there appear to be marked differences between short-acting and long-acting β_2-receptor agonists.[185] We will first review studies with short acting β_2-receptor agonists and thereafter studies with long-acting β_2-receptor agonists.

Short-acting β_2-receptor agonists

Despite the above mentioned anti-inflammatory effects of β_2-receptor agonists in 'in vitro' studies or when given as single dose, regular treatment with short-acting β_2-receptor agonists as single treatment does not appear to have any beneficial effect on inflammatory processes in asthma.[186] In contrast, increases in both the early and late asthmatic response to allergen have been found after regular treatment with a short-acting β_2-receptor agonist.[187,188] In addition, an increase in airway inflammation, as reflected by a higher percentage of sputum eosinophils, has been observed after regular treatment with the short-acting β_2-receptor agonist terbutaline 1 mg q.i.d. when compared to treatment solely with

ipratropiumbromide for relief of symptoms.[189] In the same study, treatment with a combination of terbutaline and budesonide provided conflicting outcomes. On the one hand, there was no significant difference in sputum eosinophils with combined treatment compared with budesonide alone. On the other hand, addition of terbutaline appeared to negate the budesonide-induced improvement in bronchial hyperresponsiveness to hypertonic saline. In agreement with these negative effects of short-acting β_2-receptor agonists, Taylor and colleagues found that regular treatment with a short acting β_2-receptor agonist for 24 weeks resulted in a lower FEV_1 and a higher level of bronchial hyperresponsiveness than "as needed" treatment.[190] However, there is controversy in this area, since these findings could not be reproduced in a later study.[186,191] Thus, despite their anti-inflammatory effects in 'in vitro' studies, regular treatment with short-acting β_2-receptor agonists does not seem to have an anti-inflammatory effect 'in vivo'.

A possible explanation for this discrepancy could be that subsensitivity to the effect of β_2-receptor agonists develops after regular use.[192] Several molecular mechanisms are involved in desensitization of β_2-receptors.[193] Short-term desensitization involves phosphorylation of the receptor, which results in uncoupling from the stimulatory G-protein Gs by two kinases, protein kinase A, and β-adrenergic receptor kinase (βARK).[194,195] Longer-term desensitization includes downregulation of the surface receptor, a process that involves internalization of the receptor and its subsequent degradation.

In agreement with the above, recent 'in vivo' studies in humans using positron emission tomography with a radioactive labeled β_2-blocker (S-[^{11}C]CGP12177) have demonstrated an apparent reduction in pulmonary β_2-receptor density after 2 weeks treatment with an inhaled β_2-receptor agonist.[196] In addition, it has been found that subsensitivity to the bronchoprotective effect of the short-acting β_2-receptor agonist terbutaline against AMP challenge develops when asthma patients regularly use terbutaline for one week suggesting that β_2-receptors on mast cells desensitize rapidly.[197] It has proven to be surprisingly difficult to demonstrate desensitization to the bronchodilator effects of β_2-receptor agonists in asthmatic patients, suggesting resistance of airway smooth muscle to β_2-receptor desensitization. However, a loss of bronchoprotective effect of β_2-receptor agonists could be observed after regular treatment with either salmeterol or formoterol, although the clinical relevance of this has been questioned, because some degree of bronchoprotection is maintained.[198-202] It has been suggested for several reasons that inflammatory cells may be more susceptible to β_2-receptor desensitization than airway smooth muscle cells. Firstly, inflammatory cells may have a lower rate of transcription resulting in low resynthesis of β_2-receptors.[203] Secondly, inflammatory cells may also have less receptor reserve than airway smooth muscle cells, so that when receptors are lost or uncoupled there is a concomitant loss of functional response.[204-206] Finally, there may be an increased expression of βARK in inflammatory cells, so that desensitization is greater.[207]

It has been suggested that β_2-receptors also become desensitized after exposure to inflammatory mediators, possibly via activation of PKC.[208] Indeed, it has been shown that phopholipase A_2, platelet activating factor, various arachidonic acid mediators, and IL-2 and GM-CSF impair β_2-receptor function.[209-213] In addition, there is some evidence that β_2-receptor function is impaired in circulating lymphocytes after allergen challenge.[214] Finally, studies on β_2-receptor function in untreated asthmatic subjects show a reduced β_2-receptor density and function compared to healthy control subjects.[215-217]

Long-acting β_2-receptor agonists

The long acting β_2-receptor agonists salmeterol and formoterol have been introduced into the treatment of asthma in the nineties. The introduction with long-acting β_2-receptor agonists initially caused concern due to the negative findings with short-acting β_2-receptor agonists. However, some concerns were taken away by a study of Greening and colleagues.[218] They showed that patients whose asthma was not adequately controlled with a low dose of inhaled corticosteroids (beclomethasone 400 μg daily) had little improvement in asthma control if the dose was increased (1000μg daily), but much greater improvement in symptoms and lung function if salmeterol was added. These findings were confirmed in a later study showing that addition of formoterol to budesonide more effectively controls symptoms and lung function than a four-fold increase in the dose of budesonide in patients with moderate and severe asthma, and this benefit persisted for the 12 months of the study, demonstrating that tolerance does not develop with prolonged therapy.[219] Moreover, addition of formoterol significantly reduced the number of mild and severe exacerbations when added to both a low (400 μg daily) and a high (1600 μg daily) dose of budesonide. It has been suggested that the beneficial effect of adding a long-acting β_2-receptor agonists to maintenance therapy with inhaled corticosteroids could be explained by an anti-inflammatory action of long acting β_2-receptor agonists, although this matter has been the subject of much controversy.[220] The different studies currently performed on this subject differ in the marker of inflammation examined and this makes them even more difficult to interpret. Salmeterol inhibits the rise in serum eosinophil cationic protein induced by allergen exposure.[221] On the other hand, others have shown that salmeterol does not influence the rise in urinary leukotriene E excretion 24 hours after allergen challenge.[222] Similarly, formoterol and salmeterol do not have a consistent effect on the antigen-induced increase in sputum eosinophil count.[223-225] In addition, salmeterol does not alter the cellular composition of bronchoalveolar lavage fluid when given either to steroid-treated asthmatics or to steroid-naive asthmatics.[226,227] Finally, it has been shown that 8 weeks monotherapy with formoterol significantly reduced mucosal eosinophil counts, but only in a subgroup of patient with more severe inflammation defined as $\geq$ 10 eosinophils/mm^2 biopsy tissue, whereas salmeterol when given as monotherapy for 6 weeks had no effect.[228,229] Taken together, it appears that long-acting β_2-receptor agonists, when given alone, have very limited, if any, anti-inflammatory effects in asthma.

Corticosteroids interact in a beneficial way with β_2-receptor agonists, since they prevent desensitization at various levels including enhancement of G stimulatory protein expression and an increased rate of β_2-receptor transcription.[230-233] This seems to occur not only 'in vitro', but also in humans at normally advocated doses of inhaled corticosteroids, since intranasal beclomethasone 100 μg twice daily for 3 days increases the β_2-receptor expression in nasal scrapings.[234] Alternatively, ß$_2$-agonists may potentiate the local anti-inflammatory actions of corticosteroids.[220] It may be of interest to speculate that concomitant treatment with inhaled corticosteroids is required to preserve β_2-receptor agonist function. This hypothesis could not be confirmed by a study of Aziz and colleagues, since they found that subsensitivity to the bronchoprotective effect of formoterol against AMP develops after one month even when asthma patients regularly use inhaled corticosteroids (figure 6).[197]

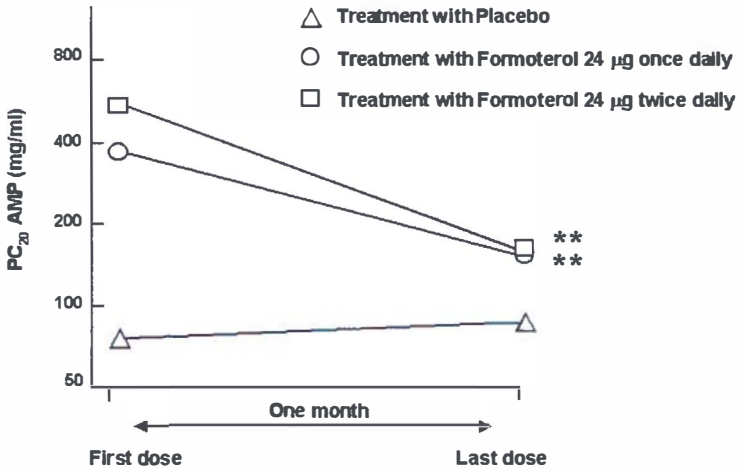


Figure 6. PC_{20} AMP after one month treatment with either placebo, formoterol 24 µg once daily, or formoterol 24 µg twice daily in addition to regular use of inhaled corticosteroids. The PC_{20} AMP was measured 12 hours after the first and last doses. The bronchoprotective effect of formoterol significantly decreased after one month treatment with both formoterol 24 µg twice daily and formoterol 24 µg once daily *(reprinted with permission from reference 197).* ** $p < 0.01$.

However, the extent of β_2-receptor desensitization is highly variable between subjects and these interindividual differences have been shown to be linked to genetic polymorphisms in the β_2-receptor.[235] Thus, it remains possible that a subgroup of asthma patients is (relatively) resistant to β_2-receptor desensitization. In this subgroup of asthma patients, adding a long-acting β_2-agonist to maintenance therapy with inhaled corticosteroids might well have an additional anti-inflammatory effect.

In summary, this review of the literature describes that it is an important research goal to identify an easily accessible non-invasive marker of airway inflammation. The PC_{20} AMP could be one such a measure that can be used for this purpose. However, thus far, the association between the severity of PC_{20} AMP and the extent of airway inflammation has not been directly investigated in the sense of actually collecting data on inflammation in sputum or airway wall biopsies. It has been suggested in several studies that adenosine and adenosine receptors may play a role in airway inflammation in asthma. Thus, it could be speculated that inhalation of AMP, and by implication adenosine, may lead to an increase in inflammation in the airways, although this matter remains to be elucidated. It has to be sorted out whether stimulation or blockade of the respective adenosine receptors will serve as an appropriate asthma treatment. This can be assessed by investigating whether the drug provides a protective effect against the allergen-induced late asthmatic response in humans. Allergen challenge responses have been extensively studied in humans. The effects of drugs on the early and late asthmatic reactions and the associated cellular influx and bronchial hyperresponsiveness provide important mechanistic insights.[236] Moreover, inhaled allergen challenges also offer the opportunity to investigate the effects of inflammatory mediators on the extent of β_2-receptor desensitization in peripheral blood lymphocytes.

AIMS OF THE STUDIES

Before presenting the studies performed in this thesis, **chapter 2** presents a review on AMP provocation as a non-invasive marker of inflammation in asthma, allergic rhinitis, and COPD.

The following aims of the studies in this thesis are formulated:

- It has been suggested in the literature that the PC_{20} AMP is more closely associated with airway inflammation in asthma than the PC_{20} methacholine or histamine. However, thus far, this has not yet been formally investigated in the sense of actually collecting data on inflammation in sputum or airway wall biopsy. In **chapter 3**, we investigate the direct association between the level of hyperresponsiveness to AMP or methacholine with inflammatory markers in sputum, blood and exhaled air in a large group of 120 asthmatic subjects.

- It has been suggested that the PC_{20} AMP is also more sensitive to changes in airway inflammation, since it improves to a greater extent after therapy with corticosteroids than PC_{20} methacholine.[116,118] However, thus far it is unknown whether this greater improvement in PC_{20} AMP is related to a better association with the reduction in airway inflammation than methacholine. In **chapter 4**, we investigate whether the steroid-induced improvement in PC_{20} AMP is more closely associated with the concomitant reduction in airway inflammation than improvement in PC_{20} methacholine.

- In recent years, there has been a tendency to attach less importance to the role of the mast cell as an effector cell in asthma. However, new findings have led to a resurgence of interest in the mast cell and support a reevaluation of its role. In particular, the discovery that mast cells are a source of Th2 type cytokines suggests that mast cell activation can contribute to orchestration of the asthmatic inflammatory response. AMP exerts its bronchoconstrictor effect via the release of inflammatory mediators from 'immunologically primed' mast cells. In **chapter 5**, we investigate whether inhalation of AMP will initiate an inflammatory response resulting in eosinophilic influx in sputum.

- Adenosine may contribute to airway inflammation in asthma, since the concentration of adenosine is elevated in the airways of asthmatic subjects and increases further after allergen challenge. In addition, it has been demonstrated in animal studies that modulation of adenosine receptors (i.e. stimulation of the A_{2A} receptor and inhibition of the adenosine A_1 receptor) has a protective effect on the late asthmatic response after allergen challenge. In **chapter 6**, we investigate whether treatment with a selective adenosine A_{2A} agonist inhibits the late asthmatic reaction in human subjects.

- Activation of β_2-receptors inhibits the release of Th2 type cytokines such as IL-5 from peripheral blood lymphocytes. However, it has been shown that β_2-receptors

desensitize after repeated stimulation. It has been suggested that inflammatory mediators may also induce desensitization of β_2-receptors, probably via activation of protein kinase C. In **chapter 7**, we investigate the β_2-adrenergic regulation of peripheral blood lymphocytes in asthmatic subjects before and 6 hours after allergen challenge.

REFERENCES

1. Jarjour NN, Kelly EA. Pathogenesis of asthma. Med Clin North Am 2002; 86:925-36.
2. Busse WW, Elias JA, Sheppard D, Banks-Schlegel S. Airway remodeling and repair. *Am J Respir Crit Care Med* 1999; 160:1035-42.
3. Reed CE. The natural history of asthma in adults: the problem of irreversibility. *J Allergy Clin Immunol* 1999; 103:539-47.
4. Backman KS, Greenberger PA, Patterson R. Airways obstruction in patients with long-term asthma consistent with 'irreversible asthma'. *Chest* 1997; 112:1234-40.
5. Bousquet J. Global initiative for asthma (GINA) and its objectives. *Clin Exp Allergy* 2000; 30 Suppl 1:2-5.
6. Kemp JP. Guidelines update: where do the new therapies fit in the management of asthma? *NHLBI and WHO Global Initiative for Asthma Drugs* 2000; 59 Suppl 1:23-8.
7. Sont JK, Han J, van Krieken JM, Evertse CE, Hooijer R, Willems LN, Sterk PJ. Relationship between the inflammatory infiltrate in bronchial biopsy specimens and clinical severity of asthma in patients treated with inhaled steroids. *Thorax* 1996; 51:496-502.
8. Boulet LP, Turcotte H, Brochu A. Persistence of airway obstruction and hyperresponsiveness in subjects with asthma remission. *Chest* 1994; 105:1024-31.
9. Sont JK, Willems LN, Bel EH, van Krieken JH, Vandenbroucke JP, Sterk PJ. Clinical control and histopathologic outcome of asthma when using airway hyperresponsiveness as an additional guide to long-term treatment. The AMPUL Study Group. *Am J Respir Crit Care Med* 1999; 159:1043-51.
10. Laitinen LA, Laitinen LA, Haahtela T. Airway mucosal inflammation even in patients with newly diagnosed asthma. *Am Rev Respir Dis* 1993; 147:697-704.
11. Sobonya RE. Quantitative structural alterations in long-standing allergic asthma. *Am Rev Respir Dis* 1984; 130:289-92.
12. Carroll NG, Elliot JG, Morton A, James AL. The structure of large and small airways in nonfatal and fatal asthma. *Am Rev Respir Dis* 1993; 147:405-10.
13. Hogg JC. Pathology of asthma. *J Allergy Clin Immunol* 1993; 92:1-5.
14. Djukanovic R, Roche WR, Wilson JW, Beasley CR, Twentyman OP, Howarth RH, Holgate ST. Mucosal inflammation in asthma. *Am Rev Respir Dis* 1990; 142:434-57.
15. Weersink EJ, Postma DS, Aalbers R, De Monchy JG. Early and late asthmatic reaction after allergen challenge. *Respir Med* 1994; 88:103-14.
16. Murray JJ, Tonnel AB, Brash AR, Roberts LJ, Gosset P, Workman R, Capron A, Oates JA. Prostaglandin D2 is released during acute allergic bronchospasm in man. *Trans Assoc Am Physicians* 1985; 98:275-80.
17. Liu MC, Hubbard WC, Proud D, Stealey BA, Galli SJ, Kagey-Sobotka A, Bleecker ER, Lichtenstein LM. Immediate and late inflammatory responses to ragweed antigen challenge of the peripheral airways in allergic asthmatics. Cellular, mediator, and permeability changes. *Am Rev Respir Dis* 1991; 144:51-8.
18. Tonnel AB, Joseph M, Gosset P, Fournier E, Capron A. Stimulation of alveolar macrophages in asthmatic patients after local provocation test. *Lancet* 1983; 1:1406-8.
19. Calhoun WJ, Reed HE, Moest DR, Stevens CA. Enhanced superoxide production by alveolar macrophages and air-space cells, airway inflammation, and alveolar macrophage density changes after segmental antigen bronchoprovocation in allergic subjects. *Am Rev Respir Dis* 1992; 145:317-25.
20. Tang RB, Chen SJ. Serum levels of eosinophil cationic protein and eosinophils in asthmatic children during a course of prednisolone therapy. *Pediatr Pulmonol* 2001; 31:121-5.
21. Fujitaka M, Kawaguchi H, Kato Y, Sakura N, Ueda K, Abe Y. Significance of the eosinophil cationic protein/eosinophil count ratio in asthmatic patients: its relationship to disease severity. *Ann Allergy Asthma Immunol* 2001; 86:323-9.
22. Jarjour NN, Calhoun WJ, Kelly EA, Gleich GJ, Schwartz LB, Busse WW. The immediate and late allergic response to segmental bronchopulmonary provocation in asthma. *Am J Respir Crit Care Med* 1997; 155:1515-21.
23. Bancalari L, Conti I, Giannessi D, Lazzerini G, Dente FL, De Caterina R, Paggiaro PL. Early increase in urinary leukotriene E4 (LTE4) is dependent on allergen dose inhaled during bronchial challenge in asthmatic subjects. *Allergy* 1999; 54:1278-85.
24. Robinson DS, Hamid QA, Bentley AM, Ying S, Kay AB, Durham SR. Activation of $CD4^+$ T cells, increased Th2-type cytokine mRNA expression, and eosinophil recruitment in bronchoalveolar lavage after allergen inhalation challenge in patients with atopic asthma. *J Allergy Clin Immunol* 1993; 92:313-24.
25. Guo CB, Liu MC, Galli SJ, Bochner BS, Kagey-Sobotka A, Lichtenstein LM. Identification of IgE-bearing cells in the late-phase response to antigen in the lung as basophils. *Am J Respir Cell Mol Biol* 1994; 10:384-90.

26. Koh YY, Dupuis R, Pollice M, Albertine KH, Fish JE, Peters SP. Neutrophils recruited to the lungs of humans by segmental antigen challenge display a reduced chemotactic response to leukotriene B4. *Am J Respir Cell Mol Biol* 1993; 8:493-9.
27. Montefort S, Gratziou C, Goulding D, Polosa R, Haskard DO, Howarth PH, Holgate ST, Carroll MP. Bronchial biopsy evidence for leukocyte infiltration and upregulation of leukocyte-endothelial cell adhesion molecules 6 hours after local allergen challenge of sensitized asthmatic airways. *J Clin Invest* 1994; 93:1411-21.
28. Granger DN, Kubes P. The microcirculation and inflammation: modulation of leukocyte- endothelial cell adhesion. *J Leukoc Biol* 1994; 55:662-75.
29. Bochner BS, Schleimer RP. The role of adhesion molecules in human eosinophil and basophil recruitment. *J Allergy Clin Immunol* 1994; 94:427-38.
30. Bousquet J, Jeffery PK, Busse WW, Johnson NM, Vignola AM. Asthma. From bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med* 2000; 161:1720-45.
31. Kuwano K, Bosken CH, Pare PD, Bai TR, Wiggs BR, Hogg JC. Small airways dimensions in asthma and in chronic obstructive pulmonary disease. *Am Rev Respir Dis* 1993; 148:1220-5.
32. James AL, Pare PD, Hogg JC. The mechanics of airway narrowing in asthma. *Am Rev Respir Dis* 1989; 139:242-6.
33. Boulet LP, Laviolette M, Turcotte H, Cartier A, Dugas M, Malo JL, Boutet M. Bronchial subepithelial fibrosis correlates with airway responsiveness to methacholine. *Chest* 1997; 112:45-52.
34. Huang J, Olivenstein R, Taha R, Hamid QA, Ludwig MS. Enhanced proteoglycan deposition in the airway wall of atopic asthmatics. *Am J Respir Crit Care Med* 1999; 160:725-9.
35. Hoshino M, Nakamura Y, Sim J, Shimojo J, Isogai S. Bronchial subepithelial fibrosis and expression of matrix metalloproteinase-9 in asthmatic airway inflammation. *J Allergy Clin Immunol* 1998; 102:783-8.
36. James AL, Maxwell PS, Pearce-Pinto G, Elliot JG, Carroll NG. The relationship of reticular basement membrane thickness to airway wall remodeling in asthma. *Am J Respir Crit Care Med* 2002; 166:1590-5.
37. Benayoun L, Druilhe A, Dombret MC, Aubier M, Pretolani M. Airway structural alterations selectively associated to severe asthma. *Am J Respir Crit Care Med* 2003.
38. Vonk JM, Jongepier H, Panhuysen CI, Schouten JP, Bleecker ER, Postma DS. Risk factors associated with the presence of irreversible airflow limitation and reduced transfer coefficient in patients with asthma after 26 years of follow-up. *Thorax* 2003;In press.
39. Ten Hacken NH, Postma DS, Timens W. Airway remodeling and long-term decline in lung function in asthma. *Curr Opin Pulm Med* 2003; 9:9-14.
40. Ulrik CS, Backer V, Dirksen A, Pedersen M, Koch C. Extrinsic and intrinsic asthma from childhood to adult age: a 10-yr follow-up. *Respir Med* 1995; 89:547-54.
41. Kelly WJ, Hudson I, Raven J, Phelan PD, Pain MC, Olinsky A. Childhood asthma and adult lung function. *Am Rev Respir Dis* 1988; 138:26-30.
42. Roorda RJ, Gerritsen J, van Aalderen WM, Schouten JP, Veltman JC, Weiss ST, Knol K. Follow-up of asthma from childhood to adulthood: influence of potential childhood risk factors on the outcome of pulmonary function and bronchial responsiveness in adulthood. *J Allergy Clin Immunol* 1994; 93:575-84.
43. Mosfeldt LE, Kaae HK, Backer V, Bach-Mortensen N, Prahl P, Koch C. Pulmonary function in adolescents with childhood asthma. *Allergy* 1993; 48:267-72.
44. Weiss ST, Tosteson TD, Segal MR, Tager IB, Redline S, Speizer FE. Effects of asthma on pulmonary function in children. A longitudinal population-based study. *Am Rev Respir Dis* 1992; 145:58-64.
45. Lange P, Parner J, Vestbo J, Schnohr P, Jensen G. A 15-year follow-up study of ventilatory function in adults with asthma. *N Engl J Med* 1998; 339:1194-200.
46. Peat JK, Woolcock AJ, Cullen K. Rate of decline of lung function in subjects with asthma. *Eur J Respir Dis* 1987; 70:171-9.
47. Burrows B, Bloom JW, Traver GA, Cline MG. The course and prognosis of different forms of chronic airways obstruction in a sample from the general population. *N Engl J Med* 1987; 317:1309-14.
48. Ulrik CS, Backer V. Nonreversible airflow obstruction in life-long nonsmokers with moderate to severe asthma. *Eur Respir J* 1999; 14:892-6.
49. Brown PJ, Greville HW, Finucane KE. Asthma and irreversible airflow obstruction. *Thorax* 1984; 39:131-6.
50. Hudon C, Turcotte H, Laviolette M, Carrier G, Boulet LP. Characteristics of bronchial asthma with incomplete reversibility of airflow obstruction. *Ann Allergy Asthma Immunol* 1997; 78:195-202.
51. Grol MH, Gerritsen J, Vonk JM, Schouten JP, Koëter GH, Rijcken B, Postma DS. Risk factors for growth and decline of lung function in asthmatic individuals up to age 42 years. A 30-year follow-up study. *Am J Respir Crit Care Med* 1999; 160:1830-7.
52. Gorski P. Global Initiative for Asthma 2002--what concerns occupational medicine. *Int J Occup Med Environ Health* 2002; 15:207-8.

53. Crimi E, Spanevello A, Neri M, Ind PW, Rossi GA, Brusasco V. Dissociation between airway inflammation and airway hyperresponsiveness in allergic asthma. *Am J Respir Crit Care Med* 1998; 157:4-9.
54. Wamboldt MZ, Bihun JT, Szefler S, Hewitt J. Perception of induced bronchoconstriction in a community sample of adolescents. *J Allergy Clin Immunol* 2000; 106:1102-7.
55. Banzett RB, Dempsey JA, O'Donnell DE, Wamboldt MZ. Symptom perception and respiratory sensation in asthma. *Am J Respir Crit Care Med* 2000; 162:1178-82.
56. de Kluijver J, Evertse CE, Schrumpf JA, van d, V, Zwinderman AH, Hiemstra PS, Rabe KF, Sterk PJ. Asymptomatic worsening of airway inflammation during low-dose allergen exposure in asthma: protection by inhaled steroids. *Am J Respir Crit Care Med* 2002; 166:294-300.
57. van den Toorn LM, Overbeek SE, de Jongste JC, Leman K, Hoogsteden HC, Prins JB. Airway inflammation is present during clinical remission of atopic asthma. *Am J Respir Crit Care Med* 2001; 164:2107-13.
58. van den Toorn LM, Prins JB, Overbeek SE, Hoogsteden HC, de Jongste JC. Adolescents in clinical remission of atopic asthma have elevated exhaled nitric oxide levels and bronchial hyperresponsiveness. *Am J Respir Crit Care Med* 2000; 162:953-7.
59. Strachan DP. The epidemiology of childhood asthma. *Allergy* 1999; 54 Suppl 49:7-11.
60. Wilson NM. Measurement of airway inflammation in asthma. *Curr Opin Pulm Med* 2002; 8:25-32.
61. Kharitonov SA, Barnes PJ. Exhaled markers of pulmonary disease. *Am J Respir Crit Care Med* 2001; 163:1693-722.
62. Jeffery PK, Laitinen LA, Venge P. Biopsy markers of airway inflammation and remodelling. *Respir Med* 2000; 94 Suppl F:S9-15.
63. Beasley R, Roche WR, Roberts JA, Holgate ST. Cellular events in the bronchi in mild asthma and after bronchial provocation. *Am Rev Respir Dis* 1989; 139:806-17.
64. Bousquet J, Chanez P, Campbell AM, Lacoste JY, Poston R, Enander I, Godard P, Michel FB. Inflammatory processes in asthma. *Int Arch Allergy Appl Immunol* 1991; 94:227-32.
65. Laitinen LA, Heino M, Laitinen LA, Kava T, Haahtela T. Damage of the airway epithelium and bronchial reactivity in patients with asthma. *Am Rev Respir Dis* 1985; 131:599-606.
66. Bousquet J, Chanez P, Lacoste JY, Barneon G, Ghavanian N, Enander I, Venge P, Ahlstedt S, Simony-Lafontaine J, Godard P, . Eosinophilic inflammation in asthma. *N Engl J Med* 1990; 323:1033-9.
67. Jarjour NN, Peters SP, Djukanovic R, Calhoun WJ. Investigative use of bronchoscopy in asthma. *Am J Respir Crit Care Med* 1998; 157:692-7.
68. Djukanovic R, Wilson JW, Britten KM, Wilson SJ, Walls AF, Roche WR, Howarth PH, Holgate ST. Quantitation of mast cells and eosinophils in the bronchial mucosa of symptomatic atopic asthmatics and healthy control subjects using immunohistochemistry. *Am Rev Respir Dis* 1990; 142:863-71.
69. Azzawi M, Bradley BL, Jeffery PK, Frew AJ, Wardlaw AJ, Knowles G, Assoufi B, Collins JV, Durham SR, Kay AB. Identification of activated T lymphocytes and eosinophils in bronchial biopsies in stable atopic asthma. *Am Rev Respir Dis* 1990; 142:1407-13.
70. Minshall EM, Leung DY, Martin RJ, Song YL, Cameron L, Ernst P, Hamid QA. Eosinophil-associated TGF-beta1 mRNA expression and airways fibrosis in bronchial asthma. *Am J Respir Cell Mol Biol* 1997; 17:326-33.
71. Wenzel SE, Trudeau JB, Westcott JY, Beam WR, Martin RJ. Single oral dose of prednisone decreases leukotriene B4 production by alveolar macrophages from patients with nocturnal asthma but not control subjects: relationship to changes in cellular influx and FEV_1. *J Allergy Clin Immunol* 1994; 94:870-81.
72. Wenzel SE, Szefler SJ, Leung DY, Sloan SI, Rex MD, Martin RJ. Bronchoscopic evaluation of severe asthma. Persistent inflammation associated with high dose glucocorticoids. *Am J Respir Crit Care Med* 1997; 156:737-43.
73. Hargreave FE, Wong BJ, Popov TA, Dolovich J. Noninvasive methods to examine the anti-inflammatory effects of drugs. *Agents Actions Suppl* 1993; 43:291-5.
74. Hargreave FE, Popov TA, Kidney J, Dolovich J. Sputum measurements to assess airway inflammation in asthma. *Allergy* 1993; 48:81-3.
75. Virchow JC, Holscher U, Virchow C, Sr. Sputum ECP levels correlate with parameters of airflow obstruction. *Am Rev Respir Dis* 1992; 146:604-6.
76. Fahy JV, Liu JT, Wong H, Boushey HA. Cellular and biochemical analysis of induced sputum from asthmatic and from healthy subjects. *Am Rev Respir Dis* 1993; 147:1126-31.
77. Fahy JV, Steiger DJ, Liu JT, Basbaum CB, Finkbeiner WE, Boushey HA. Markers of mucus secretion and DNA levels in induced sputum from asthmatic and from healthy subjects. *Am Rev Respir Dis* 1993; 147:1132-7.
78. Keatings VM, Barnes PJ. Granulocyte activation markers in induced sputum: comparison between chronic obstructive pulmonary disease, asthma, and normal subjects. *Am J Respir Crit Care Med* 1997; 155:449-53.
79. Spanevello A, Migliori GB, Satta A, Neri M, Ind PW. Sputum induction: a method to assess airway inflammation in asthma. *Monaldi Arch Chest Dis* 1995; 50:208-10.

80. Maestrelli P, Saetta M, Di Stefano A, Calcagni PG, Turato G, Ruggieri MP, Roggeri A, Mapp CE, Fabbri LM. Comparison of leukocyte counts in sputum, bronchial biopsies, and bronchoalveolar lavage. *Am J Respir Crit Care Med* 1995; 152:1926-31.

81. Fahy JV, Wong H, Liu JT, Boushey HA. Comparison of samples collected by sputum induction and bronchoscopy from asthmatic and healthy subjects. *Am J Respir Crit Care Med* 1995; 152:53-8.

82. Hoshi H, Ohno I, Honma M, Tanno Y, Yamauchi K, Tamura G, Shirato K. IL-5, IL-8 and GM-CSF immunostaining of sputum cells in bronchial asthma and chronic bronchitis. *Clin Exp Allergy* 1995; 25:720-8.

83. Fahy JV, Kim KW, Liu JT, Boushey HA. Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. *J Allergy Clin Immunol* 1995; 95:843-52.

84. O'Byrne PM, Inman MD. Induced sputum to assess airway inflammation in asthma. *Eur Respir J* 1996; 9:2435-6.

85. Pizzichini E, Pizzichini MM, Efthimiadis A, Dolovich J, Hargreave FE. Measuring airway inflammation in asthma: eosinophils and eosinophilic cationic protein in induced sputum compared with peripheral blood. *J Allergy Clin Immunol* 1997; 99:539-44.

86. Lonnkvist K, Hellman C, Lundahl J, Hallden G, Hedlin G. Eosinophil markers in blood, serum, and urine for monitoring the clinical course in childhood asthma: impact of budesonide treatment and withdrawal. *J Allergy Clin Immunol* 2001; 107:812-7.

87. Hoshino M, Nakamura Y. Relationship between activated eosinophils of the bronchial mucosa and serum eosinophil cationic protein in atopic asthma. *Int Arch Allergy Immunol* 1997; 112:59-64.

88. Meijer RJ, Kerstjens HAM, Arends LR, Kauffman HF, Koëter GH, Postma DS. Effects of inhaled fluticasone propionate and oral prednisolone on clinical and inflammatory parameters in patients with asthma. *Thorax* 1999; 54:894-9.

89. Payne DN, Wilson NM, James AL, Hablas H, Agrafioti C, Bush A. Evidence for different subgroups of difficult asthma in children. *Thorax* 2001; 56:345-50.

90. Nja F, Roksund OD, Carlsen KH. Eosinophil cationic protein (ECP) in school children living in a mountainous area of Norway: a population-based study of ECP as a tool for diagnosing asthma in children with reference values. *Allergy* 2001; 56:138-44.

91. Bahceciler NN, Basdemir D, Nursoy AM, Nuhoglu Y, Barlan IB, Basaran MM. Is serum ECP level helpful in determining discontinuation of inhaled corticosteroid therapy in asthmatic children? *Allergol Immunopathol (Madr)* 2000; 28:278-82.

92. Hoekstra MO, Grol MH, Hovenga H, Bouman K, Stijnen T, Koëter GH, Gerritsen J, Kauffman HF. Eosinophil and mast cell parameters in children with stable moderate asthma. *Pediatr Allergy Immunol* 1998; 9:143-9.

93. Artlich A, Busch T, Lewandowski K, Jonas S, Gortner L, Falke KJ. Childhood asthma: exhaled nitric oxide in relation to clinical symptoms. *Eur Respir J* 1999; 13:1396-401.

94. Massaro AF, Gaston B, Kita D, Fanta C, Stamler JS, Drazen JM. Expired nitric oxide levels during treatment of acute asthma. *Am J Respir Crit Care Med* 1995; 152:800-3.

95. Baraldi E, Dario C, Ongaro R, Scollo M, Azzolin NM, Panza N, Paganini N, Zacchello F. Exhaled nitric oxide concentrations during treatment of wheezing exacerbation in infants and young children. *Am J Respir Crit Care Med* 1999; 159:1284-8.

96. Adisesh LA, Kharitonov SA, Yates DH, Snashell DC, Newman-Taylor AJ, Barnes PJ. Exhaled and nasal nitric oxide is increased in laboratory animal allergy. *Clin Exp Allergy* 1998; 28:876-80.

97. Horvath I, Barnes PJ. Exhaled monoxides in asymptomatic atopic subjects. *Clin Exp Allergy* 1999; 29:1276-80.

98. Frank TL, Adisesh LA, Pickering AC, Morrison JF, Wright T, Francis H, Fletcher A, Frank PI, Hannaford P. Relationship between exhaled nitric oxide and childhood asthma. *Am J Respir Crit Care Med* 1998; 158:1032-6.

99. Jones SL, Kittelson J, Cowan JO, Flannery EM, Hancox RJ, McLachlan CR, Taylor DR. The predictive value of exhaled nitric oxide measurements in assessing changes in asthma control. *Am J Respir Crit Care Med* 2001; 164:738-43.

100. Kharitonov SA, Yates DH, Barnes PJ. Inhaled glucocorticoids decrease nitric oxide in exhaled air of asthmatic patients. *Am J Respir Crit Care Med* 1996; 153:454-7.

101. Jatakanon A, Kharitonov SA, Lim S, Barnes PJ. Effect of differing doses of inhaled budesonide on markers of airway inflammation in patients with mild asthma. *Thorax* 1999; 54:108-14.

102. Cockcroft DW, Ruffin RE, Dolovich J, Hargreave FE. Allergen-induced increase in non-allergic bronchial reactivity. *Clin Allergy* 1977; 7:503-13.

103. Grunberg K, Smits HH, Timmers MC, de Klerk EP, Dolhain RJ, Dick EC, Hiemstra PS, Sterk PJ. Experimental rhinovirus 16 infection. Effects on cell differentials and soluble markers in sputum in asthmatic subjects. *Am J Respir Crit Care Med* 1997; 156:609-16.

104. Schmidt D, Rabe KF. Immune mechanisms of smooth muscle hyperreactivity in asthma. *J Allergy Clin Immunol* 2000; 105:673-82.

105. Wiggs BR, Bosken CH, Pare PD, James AL, Hogg JC. A model of airway narrowing in asthma and in chronic obstructive pulmonary disease. *Am Rev Respir Dis* 1992; 145:1251-8.

106. Sterk PJ, Bel EH. Bronchial hyperresponsiveness: the need for a distinction between hypersensitivity and excessive airway narrowing. *Eur Respir J* 1989; 2:267-74.

107. Alvarez MJ, Olaguibel JM, Garcia BE, Rodriquez A, Tabar AI, Urbiola E. Airway inflammation in asthma and perennial allergic rhinitis. Relationship with nonspecific bronchial responsiveness and maximal airway narrowing. *Allergy* 2000; 55:355-62.

108. Aalbers R, De Monchy JG, Kauffman HF, Smith MJ, Hoekstra Y, Vrugt B, Timens W. Dynamics of eosinophil infiltration in the bronchial mucosa before and after the late asthmatic reaction. *Eur Respir J* 1993; 6:840-7.

109. Bradley BL, Azzawi M, Jacobson MA, Assoufi B, Collins JV, Irani AM, Schwartz LB, Durham SR, Jeffery PK, Kay AB. Eosinophils, T-lymphocytes, mast cells, neutrophils, and macrophages in bronchial biopsy specimens from atopic subjects with asthma: comparison with biopsy specimens from atopic subjects without asthma and normal control subjects and relationship to bronchial hyperresponsiveness. *J Allergy Clin Immunol* 1991; 88:661-74.

110. Finney MJ, Karlsson JA, Persson CG. Effects of bronchoconstrictors and bronchodilators on a novel human small airway preparation. *Br J Pharmacol* 1985; 85:29-36.

111. Phillips GD, Ng WH, Church MK, Holgate ST. The response of plasma histamine to bronchoprovocation with methacholine, adenosine 5'-monophosphate, and allergen in atopic nonasthmatic subjects. *Am Rev Respir Dis* 1990; 141:9-13.

112. Polosa R, Ng WH, Crimi N, Vancheri C, Holgate ST, Church MK, Mistretta A. Release of mast-cell-derived mediators after endobronchial adenosine challenge in asthma. *Am J Respir Crit Care Med* 1995; 151:624-9.

113. Phillips GD, Rafferty P, Beasley R, Holgate ST. Effect of oral terfenadine on the bronchoconstrictor response to inhaled histamine and adenosine 5'-monophosphate in non-atopic asthma. *Thorax* 1987; 42:939-45.

114. Crimi N, Palermo F, Oliveri R, Polosa R, Settinieri I, Mistretta A. Protective effects of inhaled ipratropium bromide on bronchoconstriction induced by adenosine and methacholine in asthma. *Eur Respir J* 1992; 5:560-5.

115. Benckhuijsen JA, van den Bos JW, van Velzen E, de Bruijn R, Aalbers R. Differences in the effect of allergen avoidance on bronchial hyperresponsiveness as measured by methacholine, adenosine 5'-monophosphate, and exercise in asthmatic children. *Pediatr Pulmonol* 1996; 22:147-53.

116. Weersink EJ, Douma RR, Postma DS, Koëter GH. Fluticasone propionate, salmeterol xinafoate, and their combination in the treatment of nocturnal asthma. *Am J Respir Crit Care Med* 1997; 155:1241-6.

117. Taylor DA, Jensen MW, Kanabar V, Engelstatter R, Steinijans VW, Barnes PJ, O'Connor BJ. A dose-dependent effect of the novel inhaled corticosteroid ciclesonide on airway responsiveness to adenosine-5'-monophosphate in asthmatic patients. *Am J Respir Crit Care Med* 1999; 160:237-43.

118. O'Connor BJ, Ridge SM, Barnes PJ, Fuller RW. Greater effect of inhaled budesonide on adenosine 5'-monophosphate- induced than on sodium-metabisulfite-induced bronchoconstriction in asthma. *Am Rev Respir Dis* 1992; 146:560-4.

119. Cushley MJ, Tallant N, Holgate ST. The effect of dipyridamole on histamine- and adenosine-induced bronchoconstriction in normal and asthmatic subjects. *Eur J Respir Dis* 1985; 67:185-92.

120. Crimi N, Palermo F, Oliveri R, Maccarrone C, Palermo B, Vancheri C, Polosa R, Mistretta A. Enhancing effect of dipyridamole inhalation on adenosine-induced bronchospasm in asthmatic patients. *Allergy* 1988; 43:179-83.

121. Collis MG, Hourani SM. Adenosine receptor subtypes. *Trends Pharmacol Sci* 1993; 14:360-6.

122. Walker BA, Jacobson MA, Knight DA, Salvatore CA, Weir T, Zhou D, Bai TR. Adenosine A_3 receptor expression and function in eosinophils. *Am J Respir Cell Mol Biol* 1997; 16:531-7.

123. Marquardt R, Fischer J, Kustner W. Effect of arterial hypoxia and acidosis on fibrillation threshold in the cat heart in vivo. *Z Kardiol* 1976; 65:585-9.

124. Feoktistov I, Biaggioni I. Adenosine A_{2B} receptors evoke interleukin-8 secretion in human mast cells. An enprofylline-sensitive mechanism with implications for asthma. *J Clin Invest* 1995; 96:1979-86.

125. Cronstein BN, Daguma L, Nichols D, Hutchison AJ, Williams KM. The adenosine/neutrophil paradox resolved: human neutrophils possess both A_1 and A_2 receptors that promote chemotaxis and inhibit O_2 generation, respectively. *J Clin Invest* 1990; 85:1150-7.

126. Cronstein BN, Levin RI, Philips M, Hirschhorn R, Abramson SB, Weissmann G. Neutrophil adherence to endothelium is enhanced via adenosine A_1 receptors and inhibited via adenosine A_2 receptors. *J Immunol* 1992; 148:2201-6.

127. Felsch A, Stocker K, Borchard U. Phorbol ester-stimulated adherence of neutrophils to endothelial cells is reduced by adenosine A_2 receptor agonists. *J Immunol* 1995; 155:333-8.

128. Wollner A, Wollner S, Smith JB. Acting via A_2 receptors, adenosine inhibits the upregulation of Mac-1 (Cd11b/CD18) expression on FMLP-stimulated neutrophils. *Am J Respir Cell Mol Biol* 1993; 9:179-85.

129. Fredholm BB, Zhang Y, van dP, I. Adenosine A_{2A} receptors mediate the inhibitory effect of adenosine on formyl-Met-Leu-Phe-stimulated respiratory burst in neutrophil leucocytes. *Naunyn Schmiedebergs Arch Pharmacol* 1996; 354:262-7.

130. Suzuki H, Takei M, Nakahata T, Fukamachi H. Inhibitory effect of adenosine on degranulation of human cultured mast cells upon cross-linking of Fc epsilon RI. *Biochem Biophys Res Commun* 1998; 242:697-702.

131. Polosa R, Rorke S, Holgate ST. Evolving concepts on the value of adenosine hyperresponsiveness in asthma and chronic obstructive pulmonary disease. *Thorax* 2002; 57:649-54.

132. Driver AG, Kukoly CA, Ali S, Mustafa SJ. Adenosine in bronchoalveolar lavage fluid in asthma. *Am Rev Respir Dis* 1993; 148:91-7.

133. Mann JS, Holgate ST, Renwick AG, Cushley MJ. Airway effects of purine nucleosides and nucleotides and release with bronchial provocation in asthma. *J Appl Physiol* 1986; 61:1667-76.

134. Mann JS, Holgate ST, Renwick AG, Cushley MJ. Airway effects of purine nucleosides and nucleotides and release with bronchial provocation in asthma. *J Appl Physiol* 1986; 61:1667-76.

135. Chunn JL, Young HW, Banerjee SK, Colasurdo GN, Blackburn MR. Adenosine-dependent airway inflammation and hyperresponsiveness in partially adenosine deaminase-deficient mice. *J Immunol* 2001; 167:4676-85.

136. Zhong H, Chunn JL, Volmer JB, Fozard JR, Blackburn MR. Adenosine-mediated mast cell degranulation in adenosine deaminase-deficient mice. *J Pharmacol Exp Ther* 2001; 298:433-40.

137. Fozard JR, Ellis KM, Villela Dantas MF, Tigani B, Mazzoni L. Effects of CGS 21680, a selective adenosine A_{2A} receptor agonist, on allergic airways inflammation in the rat. *Eur J Pharmacol* 2002; 438:183-8.

138. Nyce JW, Metzger WJ. DNA antisense therapy for asthma in an animal model. Nature 1997; 385:721-5.

139. Lim S, Tomita K, Carramori G, Jatakanon A, Oliver B, Keller A, Adcock IM, Chung KF, Barnes PJ. Low-dose theophylline reduces eosinophilic inflammation but not exhaled nitric oxide in mild asthma. *Am J Respir Crit Care Med* 2001; 164:273-6.

140. Morali T, Yilmaz A, Erkan F, Akkaya E, Ece F, Baran R. Efficacy of inhaled budesonide and oral theophylline in asthmatic subjects. *J Asthma* 2001; 38:673-9.

141. Sullivan P, Bekir S, Jaffar Z, Page CP, Jeffery PK, Costello J. Anti-inflammatory effects of low-dose oral theophylline in atopic asthma. *Lancet* 1994; 343:1006-8.

142. Feoktistov I, Polosa R, Holgate ST, Biaggioni I. Adenosine A_{2B} receptors: a novel therapeutic target in asthma? *Trends Pharmacol Sci* 1998; 19:148-53.

143. Klotz KN, Hessling J, Hegler J, Owman C, Kull B, Fredholm BB, Lohse MJ. Comparative pharmacology of human adenosine receptor subtypes - characterization of stably transfected receptors in CHO cells. *Naunyn Schmiedebergs Arch Pharmacol* 1998; 357:1-9.

144. Evans DJ, Taylor DA, Zetterstrom O, Chung KF, O'Connor BJ, Barnes PJ. A comparison of low-dose inhaled budesonide plus theophylline and high- dose inhaled budesonide for moderate asthma. *N Engl J Med* 1997; 337:1412-8.

145. Flint KC, Leung KB, Hudspith BN, Brostoff J, Pearce FL, Johnson NM. Bronchoalveolar mast cells in extrinsic asthma: a mechanism for the initiation of antigen specific bronchoconstriction. *Br Med J (Clin Res Ed)* 1985; 291:923-6.

146. Wardlaw AJ, Dunnette S, Gleich GJ, Collins JV, Kay AB. Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma. Relationship to bronchial hyperreactivity. *Am Rev Respir Dis* 1988; 137:62-9.

147. Metzger WJ, Zavala D, Richerson HB, Moseley P, Iwamota P, Monick MM, Sjoerdsma K, Hunninghake GW. Local allergen challenge and bronchoalveolar lavage of allergic asthmatic lungs. Description of the model and local airway inflammation. *Am Rev Respir Dis* 1987; 135:433-40.

148. Phillips GD, Holgate ST. The effect of oral terfenadine alone and in combination with flurbiprofen on the bronchoconstrictor response to inhaled adenosine 5'- monophosphate in nonatopic asthma. *Am Rev Respir Dis* 1989; 139:463-9.

149. Gibson PG, Allen CJ, Yang JP, Wong BJ, Dolovich J, Denburg JA, Hargreave FE. Intraepithelial mast cells in allergic and nonallergic asthma. Assessment using bronchial brushings. *Am Rev Respir Dis* 1993; 148:80-6.

150. Bettiol J, Radermecker M, Sele J, Henquet M, Cataldo D, Louis R. Airway mast-cell activation in asthmatics is associated with selective sputum eosinophilia. *Allergy* 1999; 54:1188-93.

151. Amin K, Ludviksdottir D, Janson C, Nettelbladt O, Bjornsson E, Roomans GM, Boman G, Seveus L, Venge P. Inflammation and structural changes in the airways of patients with atopic and nonatopic asthma. *Am J Respir Crit Care Med* 2000; 162:2295-301.

152. Ochi H, Hirani WM, Yuan Q, Friend DS, Austen KF, Boyce JA. T helper cell type 2 cytokine-mediated comitogenic responses and CCR3 expression during differentiation of human mast cells in vitro. *J Exp Med* 1999; 190:267-80.

153. Kirshenbaum AS, Goff JP, Semere T, Foster B, Scott LM, Metcalfe DD. Demonstration that human mast cells arise from a progenitor cell population that is CD34(+), c-kit(+), and expresses aminopeptidase N (CD13). *Blood* 1999; 94:2333-42.

154. Valent P, Spanblochl E, Sperr WR, Sillaber C, Zsebo KM, Agis H, Strobl H, Geissler K, Bettelheim P, Lechner K. Induction of differentiation of human mast cells from bone marrow and peripheral blood mononuclear cells by recombinant human stem cell factor/kit-ligand in long-term culture. *Blood* 1992; 80:2237-45.

155. Kanbe N, Kurosawa M, Miyachi Y, Kanbe M, Saitoh H, Matsuda H. Nerve growth factor prevents apoptosis of cord blood-derived human cultured mast cells synergistically with stem cell factor. *Clin Exp Allergy* 2000; 30:1113-20.

156. Cushley MJ, Tattersfield AE, Holgate ST. Inhaled adenosine and guanosine on airway resistance in normal and asthmatic subjects. *Br J Clin Pharmacol* 1983; 15:161-5.

157. Ulrik CS. Bronchial responsiveness to inhaled histamine in both adults with intrinsic and extrinsic asthma: the importance of prechallenge forced expiratory volume in 1 second. *J Allergy Clin Immunol* 1993; 91:120-6.

158. Bradding P, Holgate ST. The mast cell as a source of cytokines in asthma. *Ann N Y Acad Sci* 1996; 796:272-81.

159. Bradding P, Roberts JA, Britten KM, Montefort S, Djukanovic R, Mueller R, Heusser CH, Howarth PH, Holgate ST. Interleukin-4, -5, and -6 and tumor necrosis factor-alpha in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines. *Am J Respir Cell Mol Biol* 1994; 10:471-80.

160. Bradding P, Feather IH, Wilson SJ, Bardin PG, Heusser CH, Holgate ST, Howarth PH. Immunolocalization of cytokines in the nasal mucosa of normal and perennial rhinitic subjects. The mast cell as a source of IL-4, IL-5, and IL-6 in human allergic mucosal inflammation. *J Immunol* 1993; 151:3853-65.

161. Toru H, Pawankar R, Ra C, Yata J, Nakahata T. Human mast cells produce IL-13 by high-affinity IgE receptor cross- linking: enhanced IL-13 production by IL-4-primed human mast cells. *J Allergy Clin Immunol* 1998; 102:491-502.

162. Gauchat JF, Henchoz S, Mazzei G, Aubry JP, Brunner T, Blasey H, Life P, Talabot D, Flores-Romo L, Thompson J. Induction of human IgE synthesis in B cells by mast cells and basophils. *Nature* 1993; 365:340-3.

163. Pawankar R, Okuda M, Yssel H, Okumura K, Ra C. Nasal mast cells in perennial allergic rhinitics exhibit increased expression of the Fc epsilonRI, CD40L, IL-4, and IL-13, and can induce IgE synthesis in B cells. *J Clin Invest* 1997; 99:1492-9.

164. Bentley AM, Menz G, Storz C, Robinson DS, Bradley BL, Jeffery PK, Durham SR, Kay AB. Identification of T lymphocytes, macrophages, and activated eosinophils in the bronchial mucosa in intrinsic asthma. Relationship to symptoms and bronchial responsiveness. *Am Rev Respir Dis* 1992; 146:500-6.

165. Beeh KM, Ksoll M, Buhl R. Elevation of total serum immunoglobulin E is associated with asthma in nonallergic individuals. *Eur Respir J* 2000; 16:609-614.

166. Ying S, Humbert M, Barkans J, Corrigan CJ, Pfister R, Menz G, Larche M, Robinson DS, Durham SR, Kay AB. Expression of IL-4 and IL-5 mRNA and protein product by CD4+ and CD8+ T cells, eosinophils, and mast cells in bronchial biopsies obtained from atopic and nonatopic (intrinsic) asthmatics. *J Immunol* 1997; 158:3539-44.

167. Wilson SJ, Shute JK, Holgate ST, Howarth PH, Bradding P. Localization of interleukin (IL) -4 but not IL-5 to human mast cell secretory granules by immunoelectron microscopy. *Clin Exp Allergy* 2000; 30:493-500.

168. Kraneveld AD, van der Kleij HP, Kool M, van Houwelingen AH, Weitenberg AC, Redegeld FA, Nijkamp FP. Key role for mast cells in nonatopic asthma. *J Immunol* 2002; 169:2044-53.

169. O'Connor BJ, Fuller RW, Barnes PJ. Nonbronchodilator effects of inhaled beta 2 agonists. Greater protection against adenosine monophosphate- than methacholine-induced bronchoconstriction in asthma. *Am J Respir Crit Care Med* 1994; 150:381-7.

170. Egbagbe E, Pavord ID, Wilding P, Thompson-Coon J, Tattersfield AE. Adenosine monophosphate and histamine induced bronchoconstriction: repeatability and protection by terbutaline. *Thorax* 1997; 52:239-43.

171. Peters SP, Schulman ES, Schleimer RP, Macglashan DW, Jr., Newball HH, Lichtenstein LM. Dispersed human lung mast cells. Pharmacologic aspects and comparison with human lung tissue fragments. *Am Rev Respir Dis* 1982; 126:1034-9.

172. Church MK, Hiroi J. Inhibition of IgE-dependent histamine release from human dispersed lung mast cells by anti-allergic drugs and salbutamol. *Br J Pharmacol* 1987; 90:421-9.

173. Howarth PH, Durham SR, Lee TH, Kay AB, Church MK, Holgate ST. Influence of albuterol, cromolyn sodium and ipratropium bromide on the airway and circulating mediator responses to allergen bronchial provocation in asthma. *Am Rev Respir Dis* 1985; 132:986-92.

174. Dent G, Giembycz MA, Evans PM, Rabe KF, Barnes PJ. Suppression of human eosinophil respiratory burst and cyclic AMP hydrolysis by inhibitors of type IV phosphodiesterase: interaction with the beta adrenoceptor agonist albuterol. *J Pharmacol Exp Ther* 1994; 271:1167-74.

175. Munoz NM, Vita AJ, Neeley SP, McAllister K, Spaethe SM, White SR, Leff AR. Beta adrenergic modulation of formyl-methionine-leucine-phenylalanine- stimulated secretion of eosinophil peroxidase and leukotriene C4. *J Pharmacol Exp Ther* 1994; 268:139-43.

176. Rabe KF, Giembycz MA, Dent G, Perkins RS, Evans PM, Barnes PJ. Salmeterol is a competitive antagonist at beta-adrenoceptors mediating inhibition of respiratory burst in guinea-pig eosinophils. *Eur J Pharmacol* 1993; 231:305-8.

177. Galant SP, Duriseti L, Underwood S, Allred S, Insel PA. Beta adrenergic receptors of polymorphonuclear particulates in bronchial asthma. *J Clin Invest* 1980; 65:577-85.

178. Busse WW, Sosman JM. Isoproterenol inhibition of isolated human neutrophil function. *J Allergy Clin Immunol* 1984; 73:404-10.

179. Spoelstra FM, Postma DS, Hovenga H, Noordhoek JA, Kauffman HF. Additive anti-inflammatory effect of formoterol and budesonide on human lung fibroblasts. *Thorax* 2002; 57:237-41.

180. Staples KJ, Bergmann M, Tomita K, Houslay MD, McPhee I, Barnes PJ, Giembycz MA, Newton R. Adenosine 3',5'-cyclic monophosphate (cAMP)-dependent inhibition of IL- 5 from human T lymphocytes is not mediated by the cAMP-dependent protein kinase A. *J Immunol* 2001; 167:2074-80.

181. Erjefalt I, Persson CG. Long duration and high potency of antiexudative effects of formoterol in guinea-pig tracheobronchial airways. *Am Rev Respir Dis* 1991; 144:788-91.

182. Bolton PB, Lefevre P, McDonald DM. Salmeterol reduces early- and late-phase plasma leakage and leukocyte adhesion in rat airways. *Am J Respir Crit Care Med* 1997; 155:1428-35.

183. Bowden JJ, Sulakvelidze I, McDonald DM. Inhibition of neutrophil and eosinophil adhesion to venules of rat trachea by beta 2-adrenergic agonist formoterol. *J Appl Physiol* 1994; 77:397-405.

184. Baluk P, McDonald DM. The beta 2-adrenergic receptor agonist formoterol reduces microvascular leakage by inhibiting endothelial gap formation. *Am J Physiol* 1994; 266:L461-L468.

185. Kips JC, Pauwels RA. Long-acting inhaled beta(2)-agonist therapy in asthma. *Am J Respir Crit Care Med* 2001; 164:923-32.

186. Walters EH, Walters J. Inhaled short acting beta2-agonist use in asthma: regular vs as needed treatment. *Cochrane Database Syst Rev* 2000;CD001285.

187. Swystun VA, Gordon JR, Davis EB, Zhang X, Cockcroft DW. Mast cell tryptase release and asthmatic responses to allergen increase with regular use of salbutamol. *J Allergy Clin Immunol* 2000; 106:57-64.

188. Gauvreau GM, Jordana M, Watson RM, Cockroft DW, O'Byrne PM. Effect of regular inhaled albuterol on allergen-induced late responses and sputum eosinophils in asthmatic subjects. *Am J Respir Crit Care Med* 1997; 156:1738-45.

189. Aldridge RE, Hancox RJ, Robin TD, Cowan JO, Winn MC, Frampton CM, Ian TG. Effects of terbutaline and budesonide on sputum cells and bronchial hyperresponsiveness in asthma. *Am J Respir Crit Care Med* 2000; 161:1459-64.

190. Taylor DR, Sears MR, Herbison GP, Flannery EM, Print CG, Lake DC, Yates DM, Lucas MK, Li Q. Regular inhaled beta agonist in asthma: effects on exacerbations and lung function. *Thorax* 1993; 48:134-8.

191. Sears MR, Taylor DR, Print CG, Lake DC, Li Q, Flannery EM, Yates DM, Lucas MK, Herbison GP. Regular inhaled beta-agonist treatment in bronchial asthma. *Lancet* 1990; 336:1391-6.

192. Barnes PJ. Beta-adrenergic receptors and their regulation. *Am J Respir Crit Care Med* 1995; 152:838-60.

193. Ferguson SS, Zhang J, Barak LS, Caron MG. Molecular mechanisms of G protein-coupled receptor desensitization and resensitization. *Life Sci* 1998; 62:1561-5.

194. Inglese J, Freedman NJ, Koch WJ, Lefkowitz RJ. Structure and mechanism of the G protein-coupled receptor kinases. *J Biol Chem* 1993; 268:23735-8.

195. Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, Caron MG, Lefkowitz RJ. Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science* 1999; 283:655-61.

196. Hayes MJ, Qing F, Rhodes CG, Rahman SU, Ind PW, Sriskandan S, Jones T, Hughes JM. In vivo quantification of human pulmonary beta-adrenoceptors: effect of beta-agonist therapy. *Am J Respir Crit Care Med* 1996; 154:1277-83.

197. Aziz I, Tan KS, Hall IP, Devlin MM, Lipworth BJ. Subsensitivity to bronchoprotection against adenosine monophosphate challenge following regular once-daily formoterol. *Eur Respir J* 1998; 12:580-4.

198. Lipworth BJ, Aziz I. A high dose of albuterol does not overcome bronchoprotective subsensitivity in asthmatic subjects receiving regular salmeterol or formoterol. *J Allergy Clin Immunol* 1999; 103:88-92.

199. Bhagat R, Kalra S, Swystun VA, Cockcroft DW. Rapid onset of tolerance to the bronchoprotective effect of salmeterol. *Chest* 1995; 108:1235-9.

200. Lipworth BJ, Tan S, Devlin MM, Aiken T, Baker R, Hendrick D. Effects of treatment with formoterol on bronchoprotection against methacholine. *Am J Med* 1998; 104:431-8.

201. Boulet LP, Cartier A, Milot J, Cote J, Malo JL, Laviolette M. Tolerance to the protective effects of salmeterol on methacholine- induced bronchoconstriction: influence of inhaled corticosteroids. *Eur Respir J* 1998; 11:1091-7.

202. van der MT, Postma DS, Turner MO, Jong BM, Malo JL, Chapman KR, Grossman R, de Graaff CS, Riemersma RA, Sears MR. Effects of the long acting beta agonist formoterol on asthma control in asthmatic patients using inhaled corticosteroids. The Netherlands and Canadian Formoterol Study Investigators. *Thorax* 1997; 52:535-9.

203. Barnes PJ. Effect of beta-agonists on inflammatory cells. J Allergy Clin Immunol 1999; 104:S10-S17.

204. Tattersfield AE. Tolerance to beta-agonists. *Bull Eur Physiopathol Respir* 1985; 21:1s-5s.

205. Nishikawa M, Mak JC, Shirasaki H, Barnes PJ. Differential down-regulation of pulmonary beta 1- and beta 2- adrenoceptor messenger RNA with prolonged in vivo infusion of isoprenaline. *Eur J Pharmacol* 1993; 247:131-8.

206. Nishikawa M, Mak JC, Shirasaki H, Harding SE, Barnes PJ. Long-term exposure to norepinephrine results in down-regulation and reduced mRNA expression of pulmonary beta-adrenergic receptors in guinea pigs. *Am J Respir Cell Mol Biol* 1994; 10:91-9.

207. McGraw DW, Liggett SB. Heterogeneity in beta-adrenergic receptor kinase expression in the lung accounts for cell-specific desensitization of the beta2-adrenergic receptor. *J Biol Chem* 1997; 272:7338-44.

208. Pitcher J, Lohse MJ, Codina J, Caron MG, Lefkowitz RJ. Desensitization of the isolated beta 2-adrenergic receptor by beta- adrenergic receptor kinase, cAMP-dependent protein kinase, and protein kinase C occurs via distinct molecular mechanisms. *Biochemistry* 1992; 31:3193-7.

209. Taki F, Takagi K, Satake T, Sugiyama S, Ozawa T. The role of phospholipase in reduced beta-adrenergic responsiveness in experimental asthma. *Am Rev Respir Dis* 1986; 133:362-6.

210. Agrawal DK, Townley RG. Effect of platelet-activating factor on beta-adrenoceptors in human lung. *Biochem Biophys Res Commun* 1987; 143:1-6.

211. Barnes PJ, Grandordy BM, Page CP, Rhoden KJ, Robertson DN. The effect of platelet activating factor on pulmonary beta- adrenoceptors. *Br J Pharmacol* 1987; 90:709-15.

212. Raaijmakers JA, Beneker C, van Geffen EC, Meisters TM, Pover P. Inflammatory mediators and beta-adrenoceptor function. *Agents Actions* 1989; 26:45-7.

213. Folkerts G, Nijkamp FP, Van Oosterhout AJ. Induction in guinea-pigs of airway hyperreactivity and decreased lung beta-adrenoceptor number by 15-hydroperoxy-arachidonic acid. *Br J Pharmacol* 1983; 80:597-9.

214. Meurs H, Kauffman HF, Koëter GH, Timmermans A, De Vries K. Regulation of the beta-receptor-adenylate cyclase system in lymphocytes of allergic patients with asthma: possible role for protein kinase C in allergen-induced nonspecific refractoriness of adenylate cyclase. *J Allergy Clin Immunol* 1987; 80:326-39.

215. Kariman K. Beta-adrenergic receptor binding in lymphocytes from patients with asthma. *Lung* 1980; 158:41-51.

216. Brooks SM, McGowan K, Bernstein IL, Altenau P, Peagler J. Relationship between numbers of beta adrenergic receptors in lymphocytes and disease severity in asthma. *J Allergy Clin Immunol* 1979; 63:401-6.

217. Gamboa PM, Oehling A, Sanz ML, Castillo JG. Decrease of beta-receptors in asthmatic and rhinitic patients. *Allergol Immunopathol (Madr)* 1987; 15:65-8.

218. Greening AP, Ind PW, Northfield M, Shaw G. Added salmeterol versus higher-dose corticosteroid in asthma patients with symptoms on existing inhaled corticosteroid. Allen & Hanburys Limited UK Study Group. *Lancet* 1994; 344:219-24.

219. Pauwels RA, Lofdahl CG, Postma DS, Tattersfield AE, O'Byrne PM, Barnes PJ, Ullman A. Effect of inhaled formoterol and budesonide on exacerbations of asthma. Formoterol and Corticosteroids Establishing Therapy (FACET) International Study Group. *N Engl J Med* 1997; 337:1405-11.

220. Barnes PJ. Scientific rationale for inhaled combination therapy with long-acting beta2-agonists and corticosteroids. *Eur Respir J* 2002; 19:182-91.

221. Pedersen B, Dahl R, Larsen BB, Venge P. The effect of salmeterol on the early- and late-phase reaction to bronchial allergen and postchallenge variation in bronchial reactivity, blood eosinophils, serum eosinophil cationic protein, and serum eosinophil protein X. *Allergy* 1993; 48:377-82.

222. Taylor IK, O'Shaughnessy KM, Choudry NB, Adachi M, Palmer JB, Fuller RW. A comparative study in atopic subjects with asthma of the effects of salmeterol and salbutamol on allergen-induced bronchoconstriction, increase in airway reactivity, and increase in urinary leukotriene E4 excretion. *J Allergy Clin Immunol* 1992; 89:575-83.

223. Wong BJ, Dolovich J, Ramsdale EH, O'Byrne PM, Gontovnick L, Denburg JA, Hargreave FE. Formoterol compared with beclomethasone and placebo on allergen-induced asthmatic responses. *Am Rev Respir Dis* 1992; 146:1156-60.

224. Pizzichini MM, Kidney JC, Wong BJ, Morris MM, Efthimiadis A, Dolovich J, Hargreave FE. Effect of salmeterol compared with beclomethasone on allergen-induced asthmatic and inflammatory responses. *Eur Respir J* 1996; 9:449-55.

225. Dente FL, Bancalari L, Bacci E, Bartoli ML, Carnevali S, Cianchetti S, Di Franco A, Giannini D, Vagaggini B, Testi R, Paggiaro PL. Effect of a single dose of salmeterol on the increase in airway eosinophils induced by allergen challenge in asthmatic subjects. *Thorax* 1999; 54:622-4.

226. Gardiner PV, Ward C, Booth H, Allison A, Hendrick DJ, Walters EH. Effect of eight weeks of treatment with salmeterol on bronchoalveolar lavage inflammatory indices in asthmatics. *Am J Respir Crit Care Med* 1994; 150:1006-11.

227. Kraft M, Wenzel SE, Bettinger CM, Martin RJ. The effect of salmeterol on nocturnal symptoms, airway function, and inflammation in asthma. *Chest* 1997; 111:1249-54.

228. Wallin A, Sandstrom T, Soderberg M, Howarth PH, Lundback B, Della-Cioppa G, Wilson SJ, Judd M, Djukanovic R, Holgate ST, Lindberg A, Larssen L, Melander B. The effects of regular inhaled formoterol, budesonide, and placebo on mucosal inflammation and clinical indices in mild asthma. *Am J Respir Crit Care Med* 1999; 159:79-86.

229. Roberts JA, Bradding P, Britten KM, Walls AF, Wilson SJ, Gratziou C, Holgate ST, Howarth PH. The long-acting beta2-agonist salmeterol xinafoate: effects on airway inflammation in asthma. *Eur Respir J* 1999; 14:275-82.

230. Adcock IM, Stevens DA, Barnes PJ. Interactions of glucocorticoids and beta 2-agonists. *Eur Respir J* 1996; 9:160-8.

231. Davies AO, Lefkowitz RJ. Regulation of beta-adrenergic receptors by steroid hormones. *Annu Rev Physiol* 1984; 46:119-30.

232. Kalavantavanich K, Schramm CM. Dexamethasone potentiates high-affinity beta-agonist binding and g(s)alpha protein expression in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2000; 278:L1101-L1106.

233. Mak JC, Nishikawa M, Barnes PJ. Glucocorticosteroids increase beta 2-adrenergic receptor transcription in human lung. *Am J Physiol* 1995; 268:L41-L46.

234. Baraniuk JN, Ali M, Brody D, Maniscalco J, Gaumond E, Fitzgerald T, Wong G, Yuta A, Mak JC, Barnes PJ, Bascom R, Troost T. Glucocorticoids induce beta2-adrenergic receptor function in human nasal mucosa. *Am J Respir Crit Care Med* 1997; 155:704-10.

235. Reihsaus E, Innis M, Macintrye N. Mutations in the gene encoding for the β_2-adrenergic receptor in normal and asthmatic subjects. *Am J Respir Cell Mol Biol* 1993; 8:334-9.

236. Hansel TT, Erin EM, Barnes PJ. The allergen challenge. *Clin Exp Allergy* 2002; 32:162-7.

Chapter 2

Provocation with Adenosine 5'-Monophosphate as a marker of inflammation in asthma, allergic rhinitis and chronic obstructive pulmonary disease

M. van den Berge, H.A.M. Kerstjens, D.S. Postma

Department of Pulmonology, University Hospital Groningen, Groningen, the Netherlands

Clinical and Experimental Allergy 2002; 32:824-830

INTRODUCTION

Bronchial hyperresponsiveness (BHR) and airway inflammation are both characteristic features of asthma. Histamine and methacholine, stimuli that mainly act directly on airway smooth muscle, are generally used to measure the presence and severity of BHR. Although the presence and severity of BHR are related to smooth muscle contraction, airway inflammation also contributes to BHR.[1] Therefore, provocation with an indirect stimulus which exerts its effect mainly on 'primed' cells, thereby indirectly leading to smooth muscle contraction, may provide additional information about the present inflammatory cell numbers and cellular activation state in asthma. Adenosine is such a stimulus as it acts mainly via the release of inflammatory mediators from immunologically 'primed' mast cells.

Adenosine elicits a concentration-related bronchoconstriction in asthmatic, but not in normal individuals when administered by inhalation.[2] Commonly, the related nucleotide adenosine 5'-monophosphate (AMP) is used for practical purposes as it has much greater water solubility. Once inhaled, AMP is rapidly broken down to adenosine by the ubiquitous enzyme 5'-nucleotidase. The time to response of the airways after inhalation of AMP, and by implication adenosine, is similar to the rapid bronchoconstriction seen after histamine or methacholine challenge.[3]

At this time, there is an increasing interest in the role of adenosine as a bronchoconstrictor stimulus as it has been suggested that provocation with adenosine may be used as a non-invasive marker of airway inflammation.[4;5] The aim of the present paper is to review the literature assessing the value of AMP provocation as a diagnostic test in asthma, allergic rhinitis, and COPD.

THE MECHANISM OF ACTION BY WHICH ADENOSINE INDUCES BRONCHOCONSTRICTION

Although the exact mechanism by which adenosine exerts its effect has not yet been fully elucidated, there are several lines of evidence indicating that its main mechanism of action involves the release of inflammatory mediators from mast cells. Firstly, adenosine enhances the release of a variety of inflammatory mediators like histamine, prostaglandins, leukotrienes, and IL-8 from human mast cells 'in vitro'.[6;7] Secondly, it has been found that endobronchial instillation of AMP results in an immediate increase in the level of both histamine and the (mast cell specific) protease tryptase in bronchoalveolar lavage fluid.[8-10] The release of histamine is likely to be mast cell- rather than basophil derived, since sodium cromoglycate and albuterol have been shown to effectively inhibit AMP-induced bronchoconstriction, but have no activity against histamine release from basophils.[11-13] Thirdly, the potent and selective histamine receptor-antagonist terfenadine and astemizole have been shown to inhibit the bronchoconstrictor response to adenosine by more than 80%, suggesting that histamine is the predominant mast cell mediator involved in the airway response to this nucleotide.[14-16]

Enhancement of mast cell mediator release, although prominent, may not be the only mechanism of action of AMP. Activation of neural pathways may also contribute to the bronchoconstrictor response to adenosine.

A role for reflex cholinergic vagal pathways has been suggested through the induction of pain by adenosine in human skin.[17] In a later study, the involvement of a reflex cholinergic mechanism was made unlikely by the observation that the anticholinergic agent, ipratropium bromide, at an inhaled dose sufficient to produce a 196-fold protection against methacholine produced no significant protection against adenosine-induced bronchoconstriction.[18] There is controversy in this area, since two further studies demonstrated some protective effect.[19:20] However, in the latter two studies the protective effect of ipratropium bromide on adenosine induced bronchoconstriction was only small and was similar for histamine. Therefore the results may well have been due to functional antagonism secondary to its bronchodilator effect.

It has also been suggested that adenosine exerts an effect via contractile neuropeptides from sensory nerve endings.[21] In support of this view, it has been demonstrated that repeated bronchial challenges with inhaled bradykinin lead to a rapid loss of the bronchoconstrictor response to AMP.[22] However, inhibition of the enzyme neutral endopeptidase, which blocks the bronchoconstrictor effects of a variety of neuropeptides, did not enhance the bronchoconstrictor effects of AMP.[23] This makes it unlikely that the release of endogenous neuropeptides contributes to the bronchoconstrictor response of adenosine.

ADENOSINE RECEPTORS

At least four different adenosine receptors have been identified, namely adenosine A_1, A_{2A}, A_{2B}, and A_3 receptors. The rank order of potencies for various different adenosine antagonists argues against involvement of A_1, A_{2A}, and A_3 receptors and is entirely consistent with mediation by the A_{2B} receptor.[24] It seems unlikely that the adenylate cyclase-cAMP pathway is responsible for mast cell mediator release, since this mediator release is not inhibited by treatment of mast cells with KT5720, which completely blocks cAMP-dependent protein kinase activity.[25] Alternatively, activation of phospholipase C and the subsequent increase in intracellular calcium levels may mediate the effect of adenosine (see figure 1).[25]

Once inhaled, adenosine can also act on A_1, A_{2A}, and A_3 receptors, which have been identified on neutrophils, eosinophils, and macrophages. Their activation causes various pro- and anti- inflammatory actions: activation of adenosine A_1 receptors promotes chemotaxis of neutrophils and increases adherence of neutrophils to endothelial cells.[26-28] By contrast, activation of adenosine A_{2A} receptors reduces chemotaxis, activation and degranulation of neutrophils.[27:29:30] In addition, activation of A_{2A} receptors (in contrast to activation of A_{2B} receptors) suppresses the release of tryptase from mast cells.[31] A_3 receptors mediate inhibition of eosinophil chemotaxis when activated.[32] Other functions of A_3 receptors involve inhibition of neutrophil degranulation induced by LPS or TNF-α.

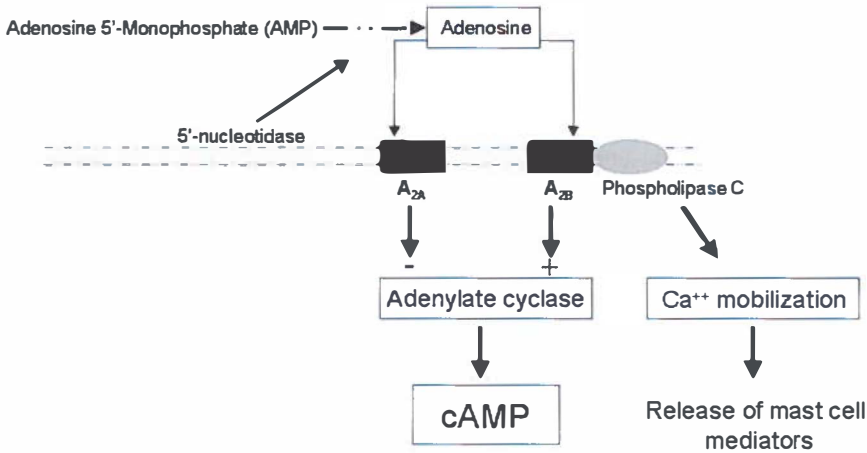


Figure 1. Mechanism of action by which adenosine interacts with human airway mast cells, thereby eliciting the release of mast cell derived inflammatory mediators.

PC_{20} TO AMP AS MARKER OF AIRWAY INFLAMMATION IN ASTHMA

The direct association between airway inflammation and bronchial hyperresponsiveness has been the subject of much controversy with almost as many negative as positive reports in the literature.[33-37] It has been suggested in several studies that the PC_{20} AMP is more closely associated with airway inflammation than the PC_{20} methacholine or PC_{20} histamine.[4;5;38] Firstly, the PC_{20} AMP improves to a larger extent after therapy with corticosteroids in asthma patients than the PC_{20} methacholine.[39-42] Secondly, the improvement of asthma after a stay of one month in a hypoallergenic environment at high altitude in Davos could be measured with the PC_{20} AMP but not the PC_{20} methacholine.[43] Thirdly, we have demonstrated for the first time directly that the PC_{20} AMP is closely associated with airway inflammation as reflected by the percentage of eosinophils in sputum and blood, whereas the association between airway inflammation and the PC_{20} methacholine was found to be only weak (see figure 2).[4] More importantly, the steroid-induced improvement of PC_{20} AMP was also more closely related to the concomitant reduction in airway inflammation than the PC_{20} methacholine.[38] Taken together, these findings indicate that the PC_{20} AMP can be a useful tool to assess the actual inflammatory state in asthma. This may be important, since it has been shown that a treatment algorithm incorporating PC_{20} methacholine as a surrogate marker of inflammation can improve the treatment of asthma and it suggests that monitoring with the PC_{20} AMP may be even more appropriate for this purpose.[33]

There are also drawbacks to the use of AMP challenge. A considerable percentage of patients is unresponsive to AMP.[44] The exact cut-off point for the PC_{20} AMP is arbitrary, since there are no population based data on this subject. However, in a previous study of Oosterhoff and coworkers, a value of 160 mg/ml was discriminatory between asthmatics and healthy controls.[45] Whether AMP unresponsive patients are clinically stable (and have low levels of airway inflammation) or unstable is yet unknown. This becomes an important

Table 1. Comparison of the level of lung function (FEV_1) and airway inflammation (i.e. eosinophils and ECP in sputum and blood in asthma patients who are unresponsive to methacholine or to AMP, before and after treatment with corticosteroids.*

	Before steroid treatment			After steroid treatment		
	PC_{20} MCh	PC_{20} AMP		PC_{20} MCh	PC_{20}AMP	
	<= 8 mg / ml	<= 160 mg / ml	> 160 mg / ml	<= 8 mg/ml	> 8 mg / ml	<= 160 mg / ml	> 160 mg / ml
	n = 120	n = 101	n = 13	n = 99	n = 14	n = 67	n = 46
FEV_1 (%pred)	78 (77 – 92)	79 (67 – 91)	92 (87 – 99)	86 (74 – 97)	92 (69 – 101)	83 (70 – 96)	91 (80 – 103)
Sputum							
eosinophils (%)	52 (2 – 11)	6 (3 – 13)	0.33 (0.17 – 1.3)	0.5 (0 – 2.3)	0.3 (0 – 1.3)	1 (0.2 – 4.5)	0.17 (0 – 0.8)
?3% eosinophils, n (%)	100 (83)	72 (73)	2 (15)	29 (30)	2 (14)	20 (31)	2 (4)
ECP (ng / l)	38 (17 – 125)	52 (21 – 127)	13.6 (4.5 – 30)	16 (7 – 47)	47 (12 – 102)	22 (9 – 57)	16 (6.4 – 44)
Blood							
eosinophils (10^9 / l)	0.37 (0.22 – 0.5)	0.35 (0.25 – 0.50)	0.16 (0.1 – 0.36)	0.26 (0.12 – 0.36)	0.2 (0.1 – 0.27)	0.22 (0.14 – 0.35)	0.18 (0.11 – 0.27)
ECP (ng / l)	16 (10 – 24)	17 (10 – 25)	10.4 (6.1 – 18)	10 (6 – 17)	10 (6 – 44)	10 (6 – 18)	9.5 (7.4 – 16)

* Values are expressed as median with interquartile ranges between brackets.

item when the severity of bronchial responsiveness to AMP is used to adjust treatment with corticosteroids in asthma patients.[33] To clarify this issue, we have reanalyzed our own database of 120 asthma patients. Inhaled corticosteroids were tapered down until these patients developed symptoms of a pending asthma exacerbation or until they had discontinued their inhaled corticosteroids completely for three weeks.[40] Thereafter, all patients received two weeks treatment with corticosteroids. Before treatment with corticosteroids, by design, no patient was unresponsive to methacholine (PC_{20} > 8 mg/ml), whereas 13 (11%) patients were unresponsive to AMP (PC_{20} > 160 mg/ml). After treatment with corticosteroids, 14 (12%) patients were unresponsive to methacholine and 46 (40%) patients were unresponsive to AMP. We found that patients who were unresponsive to AMP before treatment with corticosteroids had lower levels of airway inflammation than those who were responsive, their median percentage of sputum eosinophils being 0.33% and 6% respectively (see table 1). In addition, only 2 of the 13 AMP unresponsive patients had more than 3% eosinophils in their sputum, which is considered the upper limit of normal.[46] Furthermore, after treatment with corticosteroids, AMP unresponsive patients had also somewhat less extensive inflammation, as reflected by eosinophils and ECP in sputum and blood, than methacholine unresponsive patients (see table 1). Thus, this analysis supports the idea that a treatment algorithm incorporating the severity of PC_{20} AMP could possibly improve the treatment of asthma, although this has to be evaluated prospectively.

MAST CELLS

A possible explanation for the finding that the PC_{20} AMP is more closely associated with airway inflammation than the PC_{20} methacholine may be as follows. An overall increased inflammatory cell activity, i.e. 'primed cells' can result in an increased production of cytokines and other mediators that increase the number and activation state of mast cells (see figure 3). As a result more mast cell mediators will be released after provocation with AMP, but not with methacholine. Alternatively, the number or activation state of eosinophils may be raised as a result of an increased production of inflammatory mediators by mast cells, especially IL-5.

It is unclear whether the increase in the number and activation state of mast cells, which has consistently been reported in asthma, is a feature of allergy or of the asthmatic inflammatory process itself, since allergen exposure can increase the number and activation state of airway mast cells.[47] The finding that non-allergic patients with asthma also have a bronchoconstrictor response to AMP suggests that mast cell activation can also be a feature of the asthmatic inflammatory process rather than being dependent on the presence of allergy alone.[14;15] Indeed, both the number and activation state of mast cells were found to be increased in non-allergic patients with asthma and therefore mast cell activation and proliferation can occur under other circumstances than allergen exposure too.[48-50] It has been demonstrated 'in vitro' that growth factors for human mast cells include GM-CSF, IL-4, IL-5, IL-9, IL-10, nerve growth factor, and stem cell factor.[51;52] An increased local production of one or more of these growth factors could be responsible for mast cell accumulation and activation in the airway mucosa (see figure 3). Nevertheless, the number of mast cells is higher in allergic than in non-allergic asthma, indicating an additive effect of allergy on mast cell counts.[48] In agreement with this, inhaled adenosine was found to be

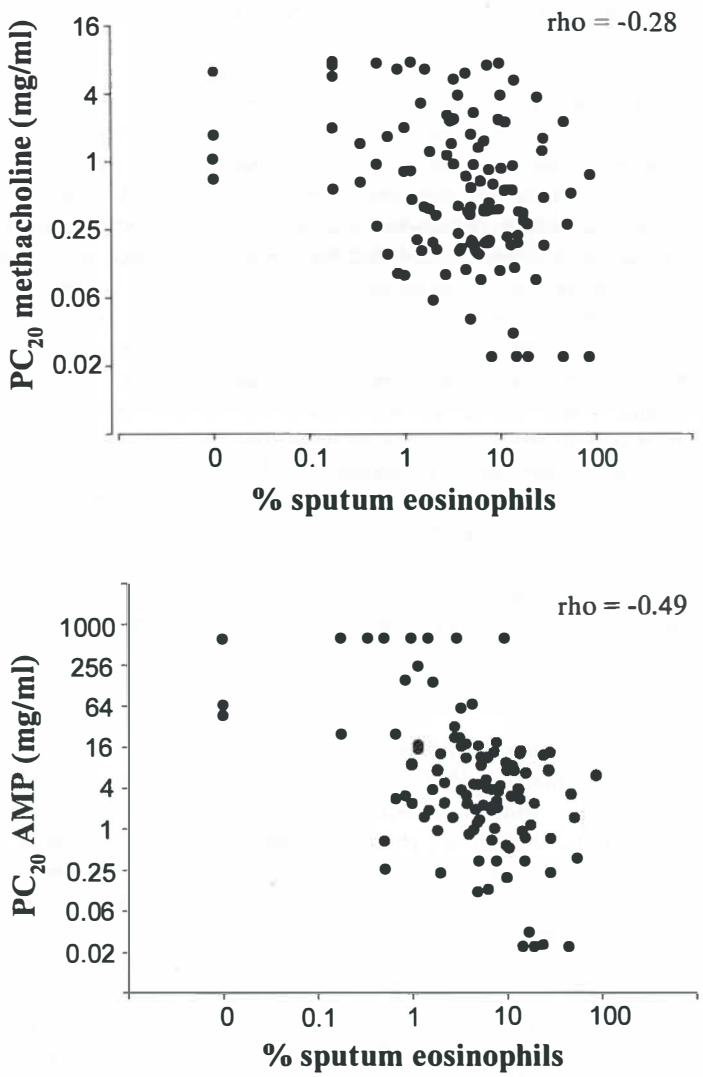


Figure 2. Unadjusted relationship between PC_{20} methacholine and PC_{20} AMP with the percentage of sputum eosinophils *(reprinted with permission from reference 4)*.

three times more potent in patients with allergic asthma than in patients with non-allergic asthma, whereas the degree of bronchial hyperresponsiveness to a direct stimulus did not differ between both groups.[2;53]

In recent years, there has been a tendency to attach less importance to the role of the mast cell as an effector cell in asthma. However, new findings have led to a resurgence of interest in the mast cell and support a reevaluation of its role. In particular, the discovery that mast cells are a source of cytokines has suggested new ways in which mast cell activation could also participate in more persistent and even chronic inflammatory responses.[54-56] For example, it has been demonstrated that the ability to produce IL-4 and IL-13 enables mast cells to stimulate IgE secretion by B cells (see figure 3).[57-59] This may explain the finding that, although the concentration of serum total IgE falls within the normal range in patients with intrinsic asthma, it is still higher than in non-allergic subjects without asthma.[60] In support of the notion that mast cells can contribute to IgE concentrations in intrinsic asthma, it was found that the level of IL-4 is elevated in these patients and (as in allergic asthma) that mast cells express a significant proportion of this cytokine.[61-63] Interestingly a higher concentration of serum total IgE is associated with a lower lung function in asthma patients, irrespective of atopic status.[61] Taken together, these observations have obvious implications for the role of mast cells in both allergic and intrinsic asthma. Provocation with AMP may therefore become a useful tool for further exploration of the role of mast cells in asthma, since there is now convincing evidence that the airway response to this nucleotide is an index of mast cell number or activity.

ALLERGIC RHINITIS

Polosa and colleagues recently demonstrated, similar to the findings in asthma patients, that the PC_{20} AMP, but not the PC_{20} methacholine, was associated with lower airway inflammation in subjects with allergic rhinitis in whom the diagnosis of asthma was specifically excluded.[5] Although asthma and allergic rhinitis are different diseases, there are strong clinical and epidemiological associations between allergic rhinitis and extrinsic asthma. Allergic rhinitis often precedes the onset of clinical asthma and is a risk factor for the development of asthma.[64;65] In addition, hyperresponsiveness can be present in subjects with allergic rhinitis without clinical evidence of asthma.[5;66] Little is known about the factors that determine bronchial hyperresponsiveness in these subjects, but it may be due to an increased inflammatory activation state (i.e. an increase of eosinophils in biopsies and induced sputum) within the airways of these subjects.[37;67;68] It has been demonstrated in an epidemiological study that the presence of an increased inflammatory activation state as reflected by the presence of both peripheral blood eosinophilia and bronchial hyperresponsiveness increases the risk to develop asthma.[69] In this respect, the observation of Polosa and colleagues that a higher level of bronchial hyperresponsiveness to AMP is associated with an increase in the inflammatory activation state in the airways of non-asthmatic subjects with allergic rhinitis is of particular interest as it may indicate a predisposition to develop asthma. However, this hypothesis has to be confirmed in long-term prospective studies of patients with allergic rhinitis, including assessment of sputum eosinophilia and bronchial hyperresponsiveness to both AMP and methacholine.

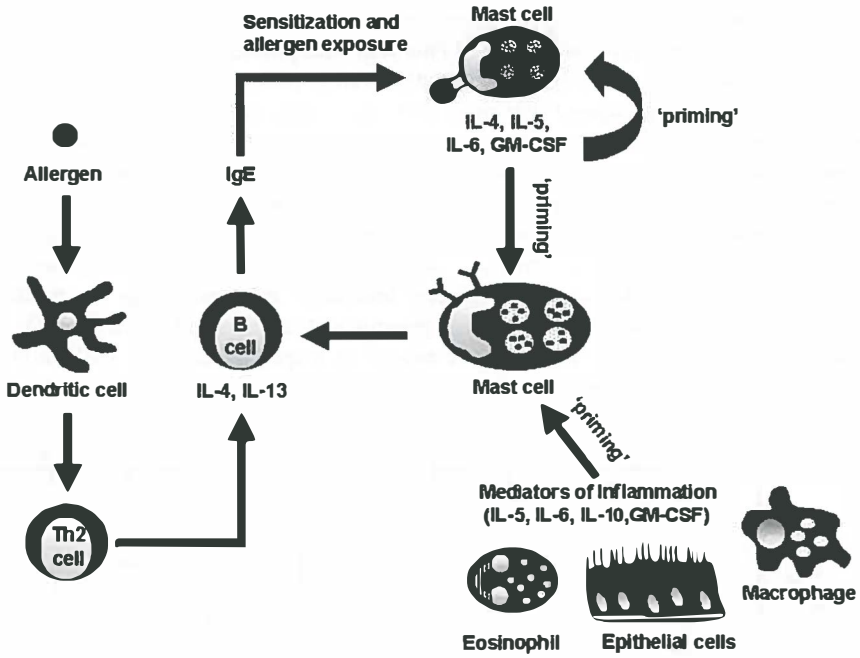


Figure 3. Schematic diagram of the mechanisms involved in mast cell priming. The mast cell can be primed by a vast array of inflammatory mediators, which can be produced during both allergic (IgE mediated) and nonallergic processes (release of inflammatory mediators during an overall increased inflammatory activity). In turn the mast is capable of influencing IgE secretion from B cells, thereby further aggravating the inflammatory process in asthma.

COPD

AMP can induce bronchoconstriction in subjects with COPD as well. Airway inflammation may play a role in AMP-induced bronchoconstriction, since hyperresponsiveness to AMP has a higher prevalence and severity in smokers than in non-smokers (or ex-smokers) with COPD who have comparable levels of hyperresponsiveness to methacholine (see figure 4).[70] This is in agreement with findings of increased concentrations of tryptase and histamine in the bronchoalveolar lavage fluid of smokers.[71;72] Although mast cells are commonly not associated with the inflammatory process in COPD, higher numbers of mast cells have been found by several groups.[71,72] It has recently been demonstrated in patients with COPD that the presence of hyperresponsiveness to AMP is accompanied by higher percentages of sputum eosinophils.[73] Airway inflammation can contribute to airflow limitation in a subgroup of patients with COPD and the presence of eosinophilic inflammation can predict benefit from corticosteroids.[74,75] Thus, it can be hypothesized that the identification of eosinophilic airway inflammation in COPD via hyperresponsiveness to AMP creates therapeutic possibilities. In support of this, the improvement of PC_{20} AMP after 6 weeks treatment with a high dose of inhaled corticosteroids was significantly correlated with the number of peripheral blood eosinophils at baseline.[73]

In patients with COPD, Renkema and colleagues recently found that a higher concentration of serum total IgE (indicating an increased inflammatory state) is associated with a lower decline in PC_{20} histamine over time.[76] This was a surprising finding, since only non-allergic patients without symptoms or signs of asthma were entered in this study. In the absence of allergy, it can be speculated that mast cells contribute to the serum IgE level, since they have the potential to influence IgE secretion from B cells and they are present in increased numbers in the airways of COPD patients.[58;59;71] If so, the increased serum IgE level in a subgroup of COPD patients might also be related to the severity of bronchial responsiveness to AMP. It can be inferred from the above that a provocation test with AMP might provide different information about the prognosis in COPD patients. It is now generally accepted that a more severe bronchial responsiveness to methacholine or histamine is associated with a poorer prognosis (i.e. faster decline in FEV_1) in COPD patients.[77;78] In contrast, a more severe bronchial responsiveness to AMP might be related to a more favourable prognosis, although this has yet to be determined.

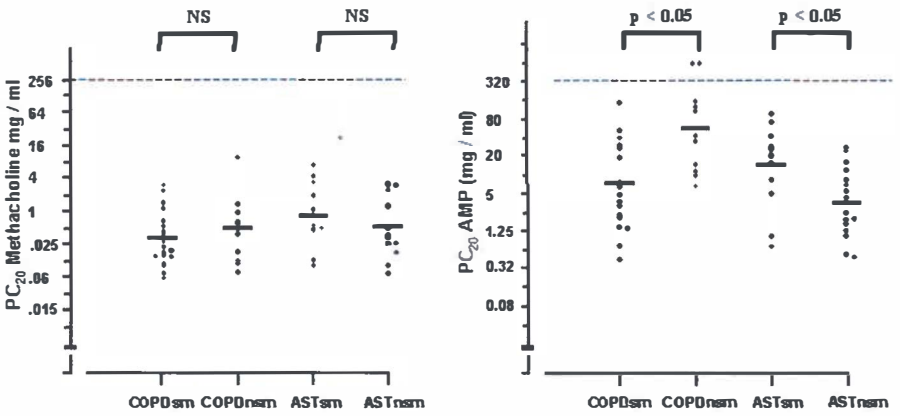


Figure 4. PC_{20} methacholine (left) and PC_{20} AMP (right) values of smoking subjects with COPD (COPDsm), nonsmoking subjects with COPD (COPDnsm), smoking asthmatics (ASTsm) and nonsmoking asthmatics (ASTnsm). Individual values are presented as closed circles. Mean values, expressed as geometric mean, are represented by horizontal bars. Dashed line indicates the highest value administered (*compiled with permission from Oosterhoff et al (references 45 and 79).*

It has been suggested that provocation with AMP can be useful as a test to differentiate between asthma and COPD. This was based on the finding that ex-smokers with COPD (but not smokers with COPD) are significantly less responsive to AMP than non-smoking asthmatic subjects, whereas the sensitivity to a direct stimulus was the same between both groups.[79] However, in this study only non-smoking asthmatics were selected and this may have lowered the PC_{20} value in the group of asthma patients, since non-smoking asthmatics are possibly less responsive to AMP due to a 'healthy smoker' effect (see figure 4).[45] In addition, patients were not allowed to take inhaled corticosteroids. This may have increased the sensitivity to AMP to a greater extent in the patients with asthma than (if at all) in the patients with COPD. Thus we feel that it is, at present, premature to suggest a role for provocation with AMP in differentiating between asthma and COPD.

CONCLUSION

AMP is an indirect stimulus to measure bronchial hyperresponsiveness as it mainly acts via the release of inflammatory mediators from mast cells. The PC_{20} AMP is more closely associated with airway inflammation than PC_{20} methacholine in asthma. In addition, the PC_{20} AMP more closely reflects changes in airway inflammation than PC_{20} methacholine either after treatment with corticosteroids or after a stay in a hypoallergenic environment. Thus, PC_{20} AMP may be a better tool to monitor airway inflammation in asthma. In addition, the PC_{20} AMP is associated with inflammation in the lower airways of patients with allergic rhinitis and without a clinical history of asthma. AMP can induce bronchoconstriction in subjects with COPD as well and in agreement with this observation higher numbers of mast cells have been found in the airways of these patients. Finally, it has been suggested that a management plan incorporating the PC_{20} AMP might improve the treatment of asthma. This hypothesis has to be sorted out in future studies.

REFERENCES

1. Busse WW. Inflammation in asthma: the cornerstone of the disease and target of therapy. *J Allergy Clin Immunol* 1998; 102:S17-S22.
2. Cushley MJ, Tattersfield AE, Holgate ST. Inhaled adenosine and guanosine on airway resistance in normal and asthmatic subjects. *Br J Clin Pharmacol* 1983; 15:161-5.
3. Phillips GD, Holgate ST. Absence of a late-phase response or increase in histamine responsiveness after bronchial provocation with adenosine 5'- monophosphate in atopic and non-atopic asthma. *Clin Sci* 1988; 75:429-36.
4. Van Den Berge M, Meijer RJ, Kerstjens HAM, De Reus DM, Koëter GH, Kauffman HF, Postma DS. PC_{20} adenosine 5'-monophosphate is more closely associated with airway inflammation in asthma than PC_{20} methacholine. *Am J Respir Crit Care Med* 2001; 163:1546-50.
5. Polosa R, Ciamarra I, Mangano G, Prosperini G, Pistorio MP, Vancheri C, Crimi N. Bronchial hyperresponsiveness and airway inflammation markers in nonasthmatics with allergic rhinitis. *Eur Respir J* 2000; 15:30-5.
6. Church MK, Holgate ST, Hughes PJ. Adenosine inhibits and potentiates IgE-dependent histamine release from human basophils by an A_2-receptor mediated mechanism. *Br J Pharmacol* 1983; 80:719-26.
7. Hughes PJ, Holgate ST, Church MK. Adenosine inhibits and potentiates IgE-dependent histamine release from human lung mast cells by an A_2-purinoceptor mediated mechanism. *Biochem Pharmacol* 1984; 33:3847-52.
8. Polosa R, Ng WH, Crimi N, Vancheri C, Holgate ST, Church MK, Mistretta A. Release of mast-cell-derived mediators after endobronchial adenosine challenge in asthma. *Am J Respir Crit Care Med* 1995; 151:624-9.
9. Phillips GD, Ng WH, Church MK, Holgate ST. The response of plasma histamine to bronchoprovocation with methacholine, adenosine 5'-monophosphate, and allergen in atopic nonasthmatic subjects. *Am Rev Respir Dis* 1990; 141:9-13.
10. Driver AG, Kukoly CA, Metzger WJ, Mustafa SJ. Bronchial challenge with adenosine causes the release of serum neutrophil chemotactic factor in asthma. *Am Rev Respir Dis* 1991; 143:1002-7.
11. Church MK. The role of basophils in asthma. Sodium cromoglycate on histamine release and content. *Clin Allergy* 1982; 12:223-8.
12. Crimi N, Palermo F, Oliveri R, Vancheri C, Polosa R, Palermo B, Maccarrone C, Mistretta A. Comparative study of the effects of nedocromil sodium (4 mg) and sodium cromoglycate (10 mg) on adenosine-induced bronchoconstriction in asthmatic subjects. *Clin Allergy* 1988; 18:367-74.
13. Phillips GD, Finnerty JP, Holgate ST. Comparative protective effect of the inhaled beta 2-agonist salbutamol (albuterol) on bronchoconstriction provoked by histamine, methacholine, and adenosine 5'-monophosphate in asthma. *J Allergy Clin Immunol* 1990; 85:755-62.
14. Phillips GD, Rafferty P, Beasley R, Holgate ST. Effect of oral terfenadine on the bronchoconstrictor response to inhaled histamine and adenosine 5'-monophosphate in non-atopic asthma. *Thorax* 1987; 42:939-45.
15. Phillips GD, Holgate ST. The effect of oral terfenadine alone and in combination with flurbiprofen on the bronchoconstrictor response to inhaled adenosine 5'- monophosphate in nonatopic asthma. *Am Rev Respir Dis* 1989; 139:463-9.
16. Rafferty P, Beasley R, Holgate ST. The contribution of histamine to immediate bronchoconstriction provoked by inhaled allergen and adenosine 5' monophosphate in atopic asthma. *Am Rev Respir Dis* 1987; 136:369-73.
17. Bleehen T, Keele CA. Observations on the algogenic actions of adenosine compounds on the human blister base preparation. *Pain* 1977; 3:367-77.
18. Mann JS, Cushley MJ, Holgate ST. Adenosine-induced bronchoconstriction in asthma. Role of parasympathetic stimulation and adrenergic inhibition. *Am Rev Respir Dis* 1985; 132:1-6.
19. Polosa R, Phillips GD, Rajakulasingam K, Holgate ST. The effect of inhaled ipratropium bromide alone and in combination with oral terfenadine on bronchoconstriction provoked by adenosine 5'- monophosphate and histamine in asthma. *J Allergy Clin Immunol* 1991; 87:939-47.
20. Crimi N, Palermo F, Oliveri R, Polosa R, Settinieri I, Mistretta A. Protective effects of inhaled ipratropium bromide on bronchoconstriction induced by adenosine and methacholine in asthma. *Eur Respir J* 1992; 5:560-5.
21. Manzini S, Ballati L. 2-Chloroadenosine induction of vagally-mediated and atropine-resistant bronchomotor responses in anaesthetized guinea-pigs. *Br J Pharmacol* 1990; 100:251-6.

22. Polosa R, Rajakulasingam K, Church MK, Holgate ST. Repeated inhalation of bradykinin attenuates adenosine 5'-monophosphate (AMP) induced bronchoconstriction in asthmatic airways. *Eur Respir J* 1992; 5:700-6.
23. Crimi N, Polosa R, Pulvirenti G, Magri S, Santonocito G, Prosperini G, Mastruzzo C, Mistretta A. Effect of an inhaled neutral endopeptidase inhibitor, phosphoramidon, on baseline airway calibre and bronchial responsiveness to bradykinin in asthma. *Thorax* 1995; 50:505-10.
24. Fozard JR, Hannon JP. Species differences in adenosine receptor-mediated bronchoconstrictor responses. *Clin Exp Allergy* 2000; 30:1213-20.
25. Marquardt DL, Walker LL. Inhibition of protein kinase A fails to alter mast cell adenosine responsiveness. *Agents Actions* 1994; 43:7-12.
26. Cronstein BN, Daguma L, Nichols D, Hutchison AJ, Williams M. The adenosine/neutrophil paradox resolved: human neutrophils possess both A_1 and A_2 receptors that promote chemotaxis and inhibit O_2 generation, respectively. *J Clin Invest* 1990; 85:1150-7.
27. Cronstein BN, Levin RI, Philips M, Hirschhorn R, Abramson SB, Weissmann G. Neutrophil adherence to endothelium is enhanced via adenosine A_1 receptors and inhibited via adenosine A_2 receptors. *J Immunol* 1992; 148:2201-6.
28. Felsch A, Stocker K, Borchard U. Phorbol ester-stimulated adherence of neutrophils to endothelial cells is reduced by adenosine A_2 receptor agonists. *J Immunol* 1995; 155:333-8.
29. Wollner A, Wollner S, Smith JB. Acting via A_2 receptors, adenosine inhibits the upregulation of Mac-1 (Cd11b/CD18) expression on FMLP-stimulated neutrophils. *Am J Respir Cell Mol Biol* 1993; 9:179-85.
30. Fredholm BB, Zhang Y, van dP, I. Adenosine A_{2A} receptors mediate the inhibitory effect of adenosine on formyl-Met-Leu-Phe-stimulated respiratory burst in neutrophil leucocytes. *Naunyn Schmiedebergs Arch Pharmacol* 1996; 354:262-7.
31. Suzuki H, Takei M, Nakahata T, Fukamachi H. Inhibitory effect of adenosine on degranulation of human cultured mast cells upon cross-linking of Fc epsilon RI. *Biochem Biophys Res Commun* 1998; 242:697-702.
32. Walker BA, Jacobson MA, Knight DA, Salvatore CA, Weir T, Zhou D, Bai TR. Adenosine A_3 receptor expression and function in eosinophils. *Am J Respir Cell Mol Biol* 1997; 16:531-7.
33. Sont JK, Han J, van Krieken JM, Evertse CE, Hooijer R, Willems LN, Sterk PJ. Relationship between the inflammatory infiltrate in bronchial biopsy specimens and clinical severity of asthma in patients treated with inhaled steroids. *Thorax* 1996; 51:496-502.
34. Aalbers R, De Monchy JG, Kauffman HF, Smith M, Hoekstra Y, Vrugt B, Timens W. Dynamics of eosinophil infiltration in the bronchial mucosa before and after the late asthmatic reaction. *Eur Respir J* 1993; 6:840-7.
35. Alvarez MJ, Olaguibel JM, Garcia BE, Rodriquez A, Tabar AI, Urbiola E. Airway inflammation in asthma and perennial allergic rhinitis. Relationship with nonspecific bronchial responsiveness and maximal airway narrowing. *Allergy* 2000; 55:355-62.
36. Crimi E, Spanevello A, Neri M, Ind PW, Rossi GA, Brusasco V. Dissociation between airway inflammation and airway hyperresponsiveness in allergic asthma. *Am J Respir Crit Care Med* 1998; 157:4-9.
37. Bradley BL, Azzawi M, Jacobson M, Assoufi B, Collins JV, Irani AM, Schwartz LB, Durham SR, Jeffery PK, Kay AB. Eosinophils, T-lymphocytes, mast cells, neutrophils, and macrophages in bronchial biopsy specimens from atopic subjects with asthma: comparison with biopsy specimens from atopic subjects without asthma and normal control subjects and relationship to bronchial hyperresponsiveness. *J Allergy Clin Immunol* 1991; 88:661-74.
38. Van Den Berge M, Kerstjens HAM, Meijer RJ, De Reus DM, Koëter GH, Kauffman HF, Postma DS. Corticosteroid-induced Improvement in the PC(20) of Adenosine Monophosphate Is More Closely Associated with Reduction in Airway Inflammation than Improvement in the PC(20) of Methacholine. *Am J Respir Crit Care Med* 2001; 164:1127-32.
39. Weersink EJ, Douma RR, Postma DS, Koëter GH. Fluticasone propionate, salmeterol xinafoate, and their combination in the treatment of nocturnal asthma. *Am J Respir Crit Care Med* 1997; 155:1241-6.
40. Meijer RJ, Kerstjens HAM, Arends LR, Kauffman HF, Koëter GH, Postma DS. Effects of inhaled fluticasone propionate and oral prednisolone on clinical and inflammatory parameters in patients with asthma. *Thorax* 1999; 54:894-9.
41. Taylor DA, Jensen MW, Kanabar V, Engelstatter R, Steinijans VW, Barnes PJ, O'Connor BJ. A dose-dependent effect of the novel inhaled corticosteroid ciclesonide on airway responsiveness to adenosine-5'-monophosphate in asthmatic patients. *Am J Respir Crit Care Med* 1999; 160:237-43.
42. O'Connor BJ, Ridge SM, Barnes PJ, Fuller RW. Greater effect of inhaled budesonide on adenosine 5'-monophosphate- induced than on sodium-metabisulfite-induced bronchoconstriction in asthma. *Am Rev Respir Dis* 1992; 146:560-4.

43. Benckhuijsen J, van den Bos JW, van Velzen E, de Bruijn R, Aalbers R. Differences in the effect of allergen avoidance on bronchial hyperresponsiveness as measured by methacholine, adenosine 5'- monophosphate, and exercise in asthmatic children. *Pediatr Pulmonol* 1996; 22:147-53.
44. Fowler SJ, Dempsey OJ, Sims EJ, Lipworth BJ. Screening for bronchial hyperresponsiveness using methacholine and adenosine monophosphate. Relationship To asthma severity and beta(2)- receptor genotype. *Am J Respir Crit Care Med* 2000; 162:1318-22.
45. Oosterhoff Y, Jansen MA, Postma DS, Koëter GH. Airway responsiveness to adenosine 5'-monophosphate in smokers and nonsmokers with atopic asthma. *J Allergy Clin Immunol* 1993; 92:773-6.
46. Gershman NH, Wong HH, Liu JT, Mahlmeister MJ, Fahy JV. Comparison of two methods of collecting induced sputum in asthmatic subjects. *Eur Respir J* 1996; 9:2448-53.
47. Wardlaw AJ, Dunnette S, Gleich GJ, Collins JV, Kay AB. Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma. Relationship to bronchial hyperreactivity. *Am Rev Respir Dis* 1988; 137:62-9.
48. Gibson PG, Allen CJ, Yang JP, Wong BJ, Dolovich J, Denburg J, Hargreave FE. Intraepithelial mast cells in allergic and nonallergic asthma. Assessment using bronchial brushings. *Am Rev Respir Dis* 1993; 148:80-6.
49. Bettiol J, Radermecker M, Sele J, Henquet M, Cataldo D, Louis R. Airway mast-cell activation in asthmatics is associated with selective sputum eosinophilia. *Allergy* 1999; 54:1188-93.
50. Amin K, Ludviksdottir D, Janson C, Nettelbladt O, Bjornsson E, Roomans GM, Boman G, Seveus L, Venge P. Inflammation and structural changes in the airways of patients with atopic and nonatopic asthma. *Am J Respir Crit Care Med* 2000; 162:2295-301.
51. Ochi H, Hirani WM, Yuan Q, Friend DS, Austen KF, Boyce JA. T helper cell type 2 cytokine-mediated comitogenic responses and CCR3 expression during differentiation of human mast cells in vitro. *J Exp Med* 1999; 190:267-80.
52. Valent P, Spanblochl E, Sperr WR, Sillaber C, Zsebo KM, Agis H, Strobl H, Geissler K, Bettelheim P, Lechner K. Induction of differentiation of human mast cells from bone marrow and peripheral blood mononuclear cells by recombinant human stem cell factor/kit-ligand in long-term culture. *Blood* 1992; 80:2237-45.
53. Ulrik CS. Bronchial responsiveness to inhaled histamine in both adults with intrinsic and extrinsic asthma: the importance of prechallenge forced expiratory volume in 1 second. *J Allergy Clin Immunol* 1993; 91:120-6.
54. Bradding P, Holgate ST. The mast cell as a source of cytokines in asthma. *Ann N Y Acad Sci* 1996; 796:272-81.
55. Bradding P, Roberts JA, Britten KM, Montefort S, Djukanovic R, Mueller R, Heusser CH, Howarth PH, Holgate ST. Interleukin-4, -5, and -6 and tumor necrosis factor-alpha in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines. *Am J Respir Cell Mol Biol* 1994; 10:471-80.
56. Bradding P, Feather IH, Wilson S, Bardin PG, Heusser CH, Holgate ST, Howarth PH. Immunolocalization of cytokines in the nasal mucosa of normal and perennial rhinitic subjects. The mast cell as a source of IL-4, IL-5, and IL-6 in human allergic mucosal inflammation. *J Immunol* 1993; 151:3853-65.
57. Toru H, Pawankar R, Ra C, Yata J, Nakahata T. Human mast cells produce IL-13 by high-affinity IgE receptor cross- linking: enhanced IL-13 production by IL-4-primed human mast cells. *J Allergy Clin Immunol* 1998; 102:491-502.
58. Gauchat JF, Henchoz S, Mazzei G, Aubry JP, Brunner T, Blasey H, Life P, Talabot D, Flores-Romo L, Thompson J. Induction of human IgE synthesis in B cells by mast cells and basophils. *Nature* 1993; 365:340-3.
59. Pawankar R, Okuda M, Yssel H, Okumura K, Ra C. Nasal mast cells in perennial allergic rhinitics exhibit increased expression of the Fc epsilonRI, CD40L, IL-4, and IL-13, and can induce IgE synthesis in B cells. *J Clin Invest* 1997; 99:1492-9.
60. Bentley AM, Menz G, Storz C, Robinson DS, Bradley B, Jeffery PK, Durham SR, Kay AB. Identification of T lymphocytes, macrophages, and activated eosinophils in the bronchial mucosa in intrinsic asthma. Relationship to symptoms and bronchial responsiveness. *Am Rev Respir Dis* 1992; 146:500-6.
61. Humbert M, Durham SR, Ying S, Kimmitt P, Barkans J, Assoufi B, Pfister R, Menz G, Robinson DS, Kay AB, Corrigan CJ. IL-4 and IL-5 mRNA and protein in bronchial biopsies from patients with atopic and nonatopic asthma: evidence against "intrinsic" asthma being a distinct immunopathologic entity. *Am J Respir Crit Care Med* 1996; 154:1497-504.
62. Ying S, Humbert M, Barkans J, Corrigan CJ, Pfister R, Menz G, Larche M, Robinson DS, Durham SR, Kay AB. Expression of IL-4 and IL-5 mRNA and protein product by CD4+ and CD8+ T cells, eosinophils, and mast cells in bronchial biopsies obtained from atopic and nonatopic (intrinsic) asthmatics. *J Immunol* 1997; 158:3539-44.
63. Wilson SJ, Shute JK, Holgate ST, Howarth PH, Bradding P. Localization of interleukin (IL) -4 but not IL-5 to human mast cell secretory granules by immunoelectron microscopy. *Clin Exp Allergy* 2000; 30:493-500.

64. Braman SS, Barrows AA, DeCotiis BA, Settipane GA, Corrao WM. Airway hyperresponsiveness in allergic rhinitis. A risk factor for asthma. *Chest* 1987; 91:671-4.
65. Settipane RJ, Hagy GW, Settipane GA. Long-term risk factors for developing asthma and allergic rhinitis: a 23-year follow-up study of college students. *Allergy Proc* 1994; 15:21-5.
66. Stevens WJ, Vermeire PA. Bronchial responsiveness to histamine and allergen in patients with asthma, rhinitis, cough. *Eur J Respir Dis* 1980; 61:203-12.
67. Djukanovic R, Lai CK, Wilson JW, Britten KM, Wilson SJ, Roche WR, Howarth PH, Holgate ST. Bronchial mucosal manifestations of atopy: a comparison of markers of inflammation between atopic asthmatics, atopic nonasthmatics and healthy controls. *Eur Respir J* 1992; 5:538-44.
68. Foresi A, Leone C, Pelucchi A, Mastropasqua B, Chetta A, D'Ippolito R, Marazzini L, Olivieri D. Eosinophils, mast cells, and basophils in induced sputum from patients with seasonal allergic rhinitis and perennial asthma: relationship to methacholine responsiveness. *J Allergy Clin Immunol* 1997; 100:58-64.
69. Jansen DF, Schouten JP, Vonk JM, Rijcken B, Timens W, Kraan J, Weiss ST, Postma DS. Smoking and airway hyperresponsiveness especially in the presence of blood eosinophilia increase the risk to develop respiratory symptoms: a 25-year follow-up study in the general adult population. *Am J Respir Crit Care Med* 1999; 160:259-64.
70. Brown HM, Storey G, George WH. Beclomethasone dipropionate: a new steroid aerosol for the treatment of allergic asthma. *Br Med J* 1972; 1:585-90.
71. Grashoff WF, Sont JK, Sterk PJ, Hiemstra PS, de Boer WI, Stolk J, Han J, van Krieken JM. Chronic obstructive pulmonary disease: role of bronchiolar mast cells and macrophages. *Am J Pathol* 1997; 151:1785-90.
72. Kalenderian R, Raju L, Roth W, Schwartz LB, Gruber B, Janoff A. Elevated histamine and tryptase levels in smokers' bronchoalveolar lavage fluid. Do lung mast cells contribute to smokers' emphysema? *Chest* 1988; 94:119-23.
73. Rutgers SR, Timens W, Tzanakis N, Kauffman HF, van der Mark TW, Koëter GH, Postma DS. Airway inflammation and hyperresponsiveness to adenosine 5'- monophosphate in chronic obstructive pulmonary disease. *Clin Exp Allergy* 2000; 30:657-62.
74. Chanez P, Vignola AM, O'Shaugnessy T, Enander I, Li D, Jeffery PK, Bousquet J. Corticosteroid reversibility in COPD is related to features of asthma. *Am J Respir Crit Care Med* 1997; 155:1529-34.
75. Pizzichini E, Pizzichini MM, Gibson P, Parameswaran K, Gleich GJ, Berman L, Dolovich J, Hargreave FE. Sputum eosinophilia predicts benefit from prednisone in smokers with chronic obstructive bronchitis. *Am J Respir Crit Care Med* 1998; 158:1511-7.
76. Renkema TE, Kerstjens HA, Schouten JP, Vonk JM, Koëter GH, Postma DS. The importance of serum IgE for level and longitudinal change in airways hyperresponsiveness in COPD. *Clin Exp Allergy* 1998; 28:1210-8.
77. Postma DS, Wempe JB, Renkema TE, van der Mark TW, Koëter GH. Hyperresponsiveness as a determinant of the outcome in chronic obstructive pulmonary disease. *Am Rev Respir Dis* 1991; 143:1458-62.
78. Tashkin DP, Altose MD, Connett JE, Kanner RE, Lee WW, Wise RA. Methacholine reactivity predicts changes in lung function over time in smokers with early chronic obstructive pulmonary disease. The Lung Health Study Research Group. *Am J Respir Crit Care Med* 1996; 153:1802-11.
79. Oosterhoff Y, de Jong JW, Jansen MA, Koëter GH, Postma DS. Airway responsiveness to adenosine 5'-monophosphate in chronic obstructive pulmonary disease is determined by smoking. *Am Rev Respir Dis* 1993; 147:553-8.

Chapter 3

PC_{20} AMP is more closely associated with airway inflammation in asthma than PC_{20} methacholine

M. van den Berge[1], R.J. Meijer[1], H.A.M. Kerstjens[1], D.M. de Reus[2], G.H. Koëter[1], H.F. Kauffman[2], D.S. Postma[1]

Departments of Pulmonology[1] and Allergology[2], University Hospital Groningen, Groningen, the Netherlands

American Journal of Respiratory and Critical Care Medicine 2001; 163:1546-1550

ABSTRACT

Introduction

Inhalation of a direct stimulus such as histamine or methacholine is generally used to measure bronchial hyperresponsiveness (BHR). Provocation with adenosine 5'-monophosphate (AMP), an indirect airway challenge, has been suggested to be a better marker of airway inflammation than direct challenges. However, so far little information on this subject is available.

Aim

To assess whether the concentration of AMP causing the FEV_1 to drop by 20% (PC_{20}) is more closely associated with inflammatory parameters in asthma than PC_{20} methacholine.

Methods

In 120 atopic asthmatics (median FEV_1 81 %predicted (pred), median age 27 years), PC_{20} methacholine and PC_{20} AMP as well as sputum induction, blood sampling, and measurement of nitric oxide in exhaled air were performed.

Results

PC_{20} methacholine was predominantly predicted by FEV_1 %pred (explained variance (ev) = 18%) with the percentage of peripheral blood monocytes being a weak additional independent predictor (total ev = 23%). By contrast, PC_{20} AMP was predominantly predicted by the percentage of eosinophils in sputum (ev = 25%), while FEV_1 %pred was only an additional independent predictor (total ev = 36%).

Conclusions

PC_{20} AMP reflects more closely the extent of airway inflammation due to asthma than PC_{20} methacholine.

INTRODUCTION

Bronchial hyperresponsiveness (BHR) and airway inflammation are both characteristic features of asthma. Histamine and methacholine, stimuli that mainly act directly on airway smooth muscle, are generally used to measure the presence and severity of BHR. Although the presence and severity of BHR are related to smooth muscle contraction, airway inflammation also contributes to BHR. Therefore indirect stimuli that exert their effect on inflammatory cells, subsequently leading to smooth muscle contraction and edema may provide additional information. Adenosine 5'-monophosphate (AMP) is such an indirect stimulus, since it has little effect on airway smooth muscle contraction 'in vitro'.[1] A major action of AMP appears to involve the release of histamine and other preformed mediators from 'primed' mast cells as AMP-induced bronchoconstriction is associated with a rise of histamine in plasma and bronchoalveolar lavage fluid.[2;3] Moreover, the concentration of AMP causing FEV_1 to drop by 20% (PC_{20}) is inhibited up to 80% by pretreatment with antihistamines.[4] AMP may have some additional actions on neural pathways as the airway response is partially attenuated by atropine and ipratropium bromide.[5;6]

Previous studies have shown that PC_{20} AMP improves to a larger extent with the use of inhaled corticosteroids than PC_{20} methacholine.[7-9] Furthermore, in a study by Aalbers and colleagues the improvement of BHR after a stay of one month in a hypoallergenic environment in Switzerland could be detected with AMP but not with methacholine.[10] These findings suggest that PC_{20} AMP is more closely associated with airway inflammation in patients with asthma than PC_{20} methacholine. However, no formal study has investigated this hypothesis so far in the sense of actually collecting data on inflammation in sputum or airway wall biopsy.

The aim of the present study was to investigate whether PC_{20} AMP is more closely associated with airway inflammation compared to PC_{20} methacholine. To this end, we tapered down inhaled corticosteroids in a large group of 120 asthmatic subjects. Thereafter, we assessed the relationship between PC_{20} methacholine as well as PC_{20} AMP with FEV_1 %predicted (pred) and inflammatory markers in sputum, blood and exhaled air.

PATIENTS AND METHODS

Patients

Patients with a diagnosis of asthma, 18-65 years old, were included if they met the following criteria: PC_{20} methacholine $\leq$ 8 mg/ml, at least one positive skin prick test out of 17 common aero-allergens, reversibility to β_2-agonist $\geq$ 9% of the predicted FEV_1, and ability to expectorate sputum after hypertonic saline inhalation.

Study design

Inhaled corticosteroids were tapered and when possible discontinued completely in 3 weeks. Patients were asked to visit the hospital on two consecutive days when they had discontinued their inhaled corticosteroids completely for three weeks, or earlier, if they

experienced (subjective) symptoms of a pending asthma exacerbation for which they felt that treatment with corticosteroids was desirable. On the first day lung function, exhaled nitric oxide (NO), blood sampling and sputum induction were performed. On the second day bronchial hyperresponsiveness was measured by methacholine challenge and after one hour a second challenge with AMP was done. The study protocol was approved by the local medical ethics committee and all participants gave their written informed consent.

Exhaled nitric oxide

Exhaled nitric oxide (NO) was measured by tidal breathing as previously described by Alving.[11]

Lung function

FEV_1 was measured with a calibrated water-sealed spirometer according to standardized guidelines as described previously.[7] PC_{20} methacholine followed by PC_{20} AMP one hour later were performed with a 2-minute tidal breathing method. Adenosine and methacholine were prepared in 0.9% saline to produce a range of concentrations from 0.04 to 320 mg /ml for adenosine and 0.038 to 8 mg/ml for methacholine.

Sputum induction and sputum processing

Sputum was induced by inhalation of hypertonic saline aerosols as previously described.[7] Fifteen minutes after salbutamol (200 μg) inhalation, hypertonic saline (3%, 4%, and 5%) was nebulized for each concentration during 7 minutes. Whole samples were processed according to the method of Fahy et al with some modifications.[7,12]

Biochemical assays

The concentration of eosinophilic cationic protein (ECP) in serum and sputum were measured using a fluoroenzyme assay, the ImmunoCAP ECP (provided by Pharmacia, Uppsala, Sweden). The concentration of interleukin-8 (IL-8) in sputum was measured using an available ELISA kit (CLB, Amsterdam).

Statistical methods

All calculations of PC_{20} were performed with the base-2 logarithm (log2) since this reflects doubling concentrations and normalizes the distribution. Patients already responding to saline were assigned a PC_{20} value half of the lowest concentration applied.[13] Patients not responding to the highest concentration of methacholine or AMP were assigned a value twice the highest concentration applied. Normality of distributions was assessed using Kolmogorov-Smirnov test. If this test resulted in a p-value < 0.05, normalization by logarithmic transformation was attempted. Correlations between variables were calculated by Pearson's correlation test in case of normal distribution or by Spearman's correlations test otherwise. To determine independent prognostic factors of PC_{20} methacholine and PC_{20} AMP multiple regression analysis was employed, in the stepwise algorithm (SPSS PC+ 9.0). In this analysis, the use of corticosteroids (yes/no), and the number of days corticosteroids had been stopped at the time of measurement were entered as covariates.

RESULTS

Patient characteristics

One hundred and twenty patients with mild to moderately severe asthma were enrolled in the study. Baseline characteristics are presented in table 1.

Table 1. Characteristics and cell count data of the study population (n=120).*

Age (yrs)	27	(18 – 56)
Gender M/F	41/79	
Smoking (%)		
Current	26	
Non-smoker	74	
FEV_1 (%pred.)	80	(15 – 110)
Reversibility (%pred)	12	(0 – 46)
PC_{20} methacholine (mg/ml)	0.47	(0.019 – 7.89)
PC_{20} AMP (mg/ml)	4.16	(0.02 – 640)
Exhaled NO (ppb)#	14.3	(1.8 – 37.1)
Sputum		
Total cell count (* 10^6)†	4.5	(0.12 – 43.4)
Macrophages (%)	44.2	(5.8 – 83.8)
Lymphocytes (%)	1.8	(0 – 9)
Neutrophils (%)	34.3	(4 – 92.2)
Eosinophils (%)	5	(0 – 88.5)
Bronchial epithelial cells (%)	2.2	(0 – 29.2)
ECP (ng/ml)	76	(1.8 – 37.1)
IL-8 (pg/ml)	2190	(164 – 15048)

* Values are expressed as median (range). †The percentages groupwise do not add up. This is due to the non-normal distributions and the expression as medians instead of means. # The reference value of exhaled nitric oxide in our laboratory is 6.0 ppb – 10 ppb.

During the steroid tapering period, 16 patients returned to the hospital earlier due to symptoms of a pending asthma exacerbation. In this study, it was mandatory that patients completely discontinued their inhaled corticosteroids for at least three weeks. From these 16 patients, 6 patients still used inhaled corticosteroids (3 patients 400 μg/day budesonide or beclomethasone, 2 patients 250 μg/day fluticasone propionate, and 1 patient 800 μg/day budesonide). The remaining 10 patients had not used inhaled corticosteroids for a median period of 12 days (range 2 - 21 days). It was not possible to measure the PC_{20} methacholine and PC_{20} AMP in all patients, due to asthma symptoms and low FEV_1 (the lowest FEV_1 was 15 %pred). Thus, we were able to measure the PC_{20} methacholine and the PC_{20} AMP in 118 (98%) and 114 (95%) patients respectively. All patients were, by design, responsive to methacholine ($PC_{20} \leq 8$ mg /ml), whereas 102 of the 114 (89%) patients were responsive to AMP ($PC_{20} \leq 320$ mg/ml). Of the 16 patients who returned to the hospital before they had completely discontinued their inhaled corticosteroids for at least three weeks, the PC_{20} AMP could not be measured in 2 patients, 13 patients were responsive to AMP, and 1 patient was not responsive to AMP.

Individual correlations of PC_{20} methacholine and PC_{20} AMP with clinical and inflammatory parameters

A high correlation for both PC_{20} methacholine and PC_{20} AMP was found with FEV_1 %pred, correlations being very comparable ($r = 0.45$, $p < 0.01$ and $r = 0.43$, $p < 0.01$ respectively) (Table 2, fig. 1). There was an inverse correlation between percentage of sputum eosinophils and PC_{20} methacholine as well as PC_{20} AMP, the correlation being stronger with PC_{20} AMP ($\rho = -0.49$, $p < 0.01$) than with PC_{20} methacholine ($\rho = -0.29$, $p < 0.01$) (fig. 2). Furthermore PC_{20} methacholine was significantly correlated with the percentage of lymphocytes and ECP per eosinophil in sputum, while PC_{20} AMP significantly correlated with ECP per eosinophil in sputum, ECP in sputum, number of eosinophils in blood, and ECP in blood.

Table 2. Correlation of clinical and inflammatory parameters with PC_{20} methacholine and PC_{20} AMP.

	PC_{20} Methacholine	PC_{20} AMP
FEV_1 %pred	r = **0.45****	r = **0.43****
Sputum cell differential, (%)		
Eosinophils†	ρ = - **0.28****	ρ = - **0.49****
Lymphocytes†	r = **0.22***	r = 0.11
Macrophages	r = 0.09	r = 0.07
Neutrophils	r = 0.09	r = 0.16
Bronchial epithelium†	r = - 0.05	r = - 0.1
Sputum ECP†	r = - 0.18	r = - **0.27****
Sputum ECP/Eosinophil†	r = **0.22***	r = **0.38****
Sputum IL-8†	r = - 0.18	r = 0.08
Blood cell differential, (10^9/ml)		
Leucocytes	r = 0.00	r = - 0.02
Neutrophils	r = 0.08	r = 0.09
Lymphocytes	r = - 0.04	r = - 0.07
Monocytes	r = 0.15	r = 0.1
Eosinophils	r = - 0.14	r = - **0.31****
Basophils†	r = 0.11	r = 0.03
Blood ECP†	r = - 0.16	r = - **0.32****
Blood ECP/eosinophil†	r = 0.08	r = 0.11
Exhaled NO	r = 0.07	r = - 0.09

*$p < 0.05$, **$p < 0.01$, †log transformed.

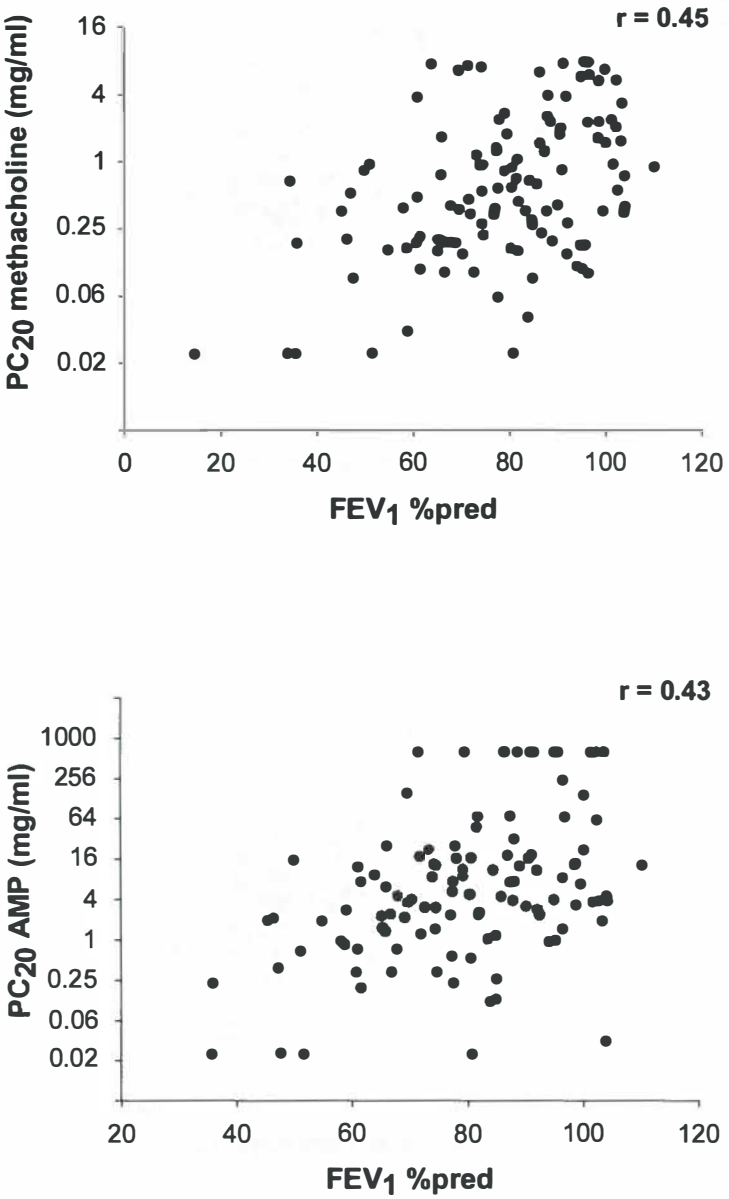


Figure 1. Unadjusted relationship between PC_{20} methacholine and PC_{20} AMP with the level of FEV_1, %pred.

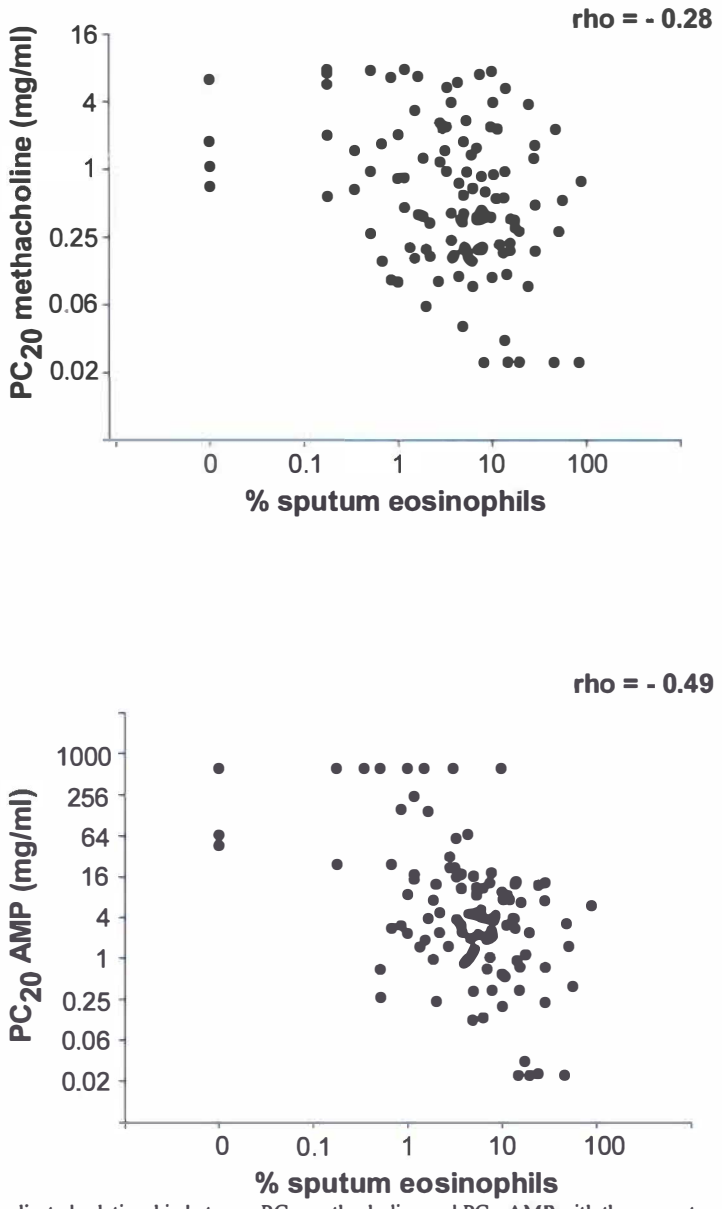


Figure 2. Unadjusted relationship between PC_{20} methacholine and PC_{20} AMP with the percentage of sputum eosinophils

Independent correlates of PC_{20} methacholine and PC_{20} AMP in a multiple linear regression model

In a multiple linear stepwise regression model, PC_{20} methacholine was predominantly predicted by FEV_1 % pred, while the number of peripheral blood monocytes was an independent additional predictor (Table 3).

Table 3. Multiple linear regression models for PC_{20} methacholine and PC_{20} AMP.*†

	PC_{20} methacholine				PC_{20} AMP	
	β#	Explained variance			β#	Explained variance
1. FEV_1 %pred	0.42	18%	1.	Sputum eosinophils (%)	-0.56	25%
2. FEV_1 %pred	0.40	23%	2.	Sputum eosinophils, (%)	-0.50	36%
Blood monocytes	0.22			FEV_1 %pred	0.39	

*Presentation of different sequential models selected by the stepwise method in SPSS-package 9.0. #Standardized partial correlation coefficient. †Model run with all variables from Table 2; the use of inhaled corticosteroids (yes/no) and the number of days patients had stopped their inhaled corticosteroids at the time of measurement were entered as covariates.

In contrast, PC_{20} AMP was predominantly predicted by the percentage eosinophils in sputum, while FEV_1 %pred was an additional independent predictor for the level of PC_{20} AMP. The total percentage explained variance was larger for PC_{20} AMP (36%) than for PC_{20} methacholine (23%). When the use of corticosteroids (yes/no), and the number of days corticosteroids had been stopped at the time of measurement were entered as covariates, their regression coefficients were not significant.

DISCUSSION

This study in a large group of asthma patients demonstrates that the severity of PC_{20} methacholine and PC_{20} AMP are both associated with baseline level of FEV_1 %pred. PC_{20} AMP provides a better reflection of airway inflammation than PC_{20} methacholine, since the percentage of sputum eosinophils explains 25% of the variance in PC_{20} AMP while it is not a significant independent predictor for PC_{20} methacholine. These results were independent of whether or not patients were still using inhaled corticosteroids at the time of measurement.

A positive association between the severity of bronchial hyperresponsiveness and the level of FEV_1 has been observed before.[14] It can be explained by the fact that a given stimulus will result in a larger bronchoconstrictor response in a subject with more severe airway obstruction than in a subject with less severe obstruction, resulting in a lower provocative concentration of the stimulus under study causing a 20% reduction in FEV_1.[15-17] Thus, it was expected that both the severity of PC_{20} methacholine and PC_{20} AMP would be associated with the level of FEV_1 %pred.

The observed association between more severe bronchial hyperresponsiveness to both PC_{20} methacholine and PC_{20} AMP and more extensive airway inflammation, i.e. the percentage

of eosinophils in sputum was to be expected. Worsening of asthma following viral infection or antigen exposure is associated with an increase in bronchial hyperresponsiveness and in case of experimental rhinovirus accompanied by an increase in sputum eosinophils and ECP.[18;19] Furthermore, bronchial hyperresponsiveness and sputum eosinophilia improve after therapy with inhaled corticosteroids.[7] Activation of different inflammatory cells leads to airway wall edema with a concomitant increase in airway wall thickness. In addition, smooth muscle cells become more sensitive to contracting stimuli, all contributing to an increase in bronchial responsiveness.[20;21]

In our study, we found a dichotomy in the factors explaining the severity of PC_{20} methacholine and PC_{20} AMP. The level of FEV_1 %pred was the most important explanatory factor for the severity of PC_{20} methacholine. Although the level of FEV_1 %pred was also a significant factor in explaining the severity of PC_{20} AMP, greater contribution was derived from the percentage of sputum eosinophils. Therefore PC_{20} AMP, which acts indirectly via the release of mediators from primed mast cells, reflects to a larger extent the cellular activation state in asthma than PC_{20} methacholine. There are two possible explanations for the finding that PC_{20} AMP is more closely associated with eosinophilic airway inflammation than PC_{20} methacholine. Firstly, an increased number of eosinophils may reflect an overall increased inflammatory activity resulting in an increased production of cytokines that stimulate mast cell chemotaxis, maturation or activation directly or indirectly.[22] Secondly it can be speculated that the exposure to aeroallergens in allergic subjects activates mast cells through IgE-dependent pathways resulting in the release of mediators which are responsible for an influx of eosinophils in sputum. We hypothesize, that if tryptase or another marker of mast cell activity was to be measurable in sputum or blood, a closer association might be demonstrable for PC_{20} AMP with sputum tryptase than for PC_{20} AMP with the percentage of sputum eosinophils.

Polosa and colleagues recently reported an association between the number of eosinophils in sputum and PC_{20} AMP, but not PC_{20} methacholine in 12 subjects with allergic rhinitis in whom the diagnosis of asthma was specifically excluded.[23] Although asthma and allergic rhinitis may be different diseases, patients with allergic rhinitis without clinical evidence of asthma have been shown to exhibit airway inflammation as reflected by an increase of eosinophils in sputum.[24] Thus, the data of Polosa et al are compatible with ours. We extended their observation in two ways. Firstly, we measured sputum ECP and showed in a simple regression that activation of eosinophils, as measured by ECP, was associated with more severe responsiveness to AMP but not to methacholine. This did, however, not contribute significantly in the multiple regression analysis. Multiple regression was not performed by Polosa. The current analysis for the first time clearly showed that PC_{20} AMP is more closely associated with eosinophils in sputum than PC_{20} methacholine.

The observation that a lower number of peripheral blood monocytes is independently associated with more severe responsiveness to methacholine was not expected. In a previous study, Sont et al also found an association between peripheral blood monocytes and bronchial hyperresponsiveness to hypertonic saline. It has been suggested that peripheral blood monocytes may play a role in immune responses.[25] To date, however, relatively little is known about the modulatory role of peripheral blood monocytes in airway inflammation. Therefore this finding is of interest and merits further investigation.

In our study, PC_{20} AMP but not PC_{20} methacholine was associated equally strongly with the number of peripheral blood eosinophils and with the concentration of ECP in serum. Peripheral blood eosinophils have been shown to be associated with the severity of symptoms, the level of FEV_1, and bronchial hyperresponsiveness to methacholine and histamine.[26-29] Furthermore, serum ECP has been shown to reflect the degree of eosinophil activation.[30] Thus, it has been suggested that both peripheral blood eosinophils and serum ECP may be indirect markers of airway inflammation in asthma.[24] However, both the number of peripheral blood eosinophils and the concentration of ECP in serum did not contribute to a better prediction of PC_{20} AMP in multiple regression analysis. The probable explanation for this finding is that the percentage of eosinophils in sputum, the number of peripheral blood eosinophils and serum ECP provide overlapping information about the inflammatory activation state in asthma.

Finally, we did not find exhaled NO to be related to either PC_{20} methacholine or PC_{20} AMP. The concentration of exhaled NO has been suggested to reflect airway inflammation. Patients with asthma have a higher concentration of exhaled NO compared to normal individuals, which is reduced after treatment with corticosteroids. However, the direct association between the concentration of exhaled NO and airway inflammation as reflected by the number of eosinophils in bronchial biopsies or sputum has been a matter of controversy.[31,32] Therefore, the absence of an association between the concentration of exhaled NO and PC_{20} AMP does not exclude PC_{20} AMP as a marker of airway inflammation.

In conclusion, the results of this study show for the first time that PC_{20} AMP is more closely associated with airway inflammation than PC_{20} methacholine. Therefore, PC_{20} AMP provides both clinicians and researchers with a non-invasive marker of disease activity. Further studies have to assess whether reduction of airway wall inflammation in asthma, e.g. by corticosteroids, is also more closely associated with improvement in PC_{20} AMP than PC_{20} methacholine.

ACKNOWLEDGEMENTS

This study was supported by grants from the University of Groningen, the University Hospital of Groningen and from GlaxoSmithKline.

We thank Pharmacia Uppsala for generously providing the kits for ECP determination.

We thank B. Dijkhuizen and J. Zonderland for their laboratory assistance.

REFERENCES

1. Finney MJ, Karlsson JA, Persson CG. Effects of bronchoconstrictors and bronchodilators on a novel human small airway preparation. *Br J Pharmacol* 1985; 85:29-36.
2. Phillips GD, Ng WH, Church MK, Holgate ST. The response of plasma histamine to bronchoprovocation with methacholine, adenosine 5'-monophosphate, and allergen in atopic nonasthmatic subjects. *Am Rev Respir Dis* 1990; 141:9-13.
3. Polosa R, Ng WH, Crimi N, Vancheri C, Holgate ST, Church MK, Mistretta A. Release of mast-cell-derived mediators after endobronchial adenosine challenge in asthma. *Am J Respir Crit Care Med* 1995; 151:624-629.
4. Phillips GD, Rafferty P, Beasley R, Holgate ST. Effect of oral terfenadine on the bronchoconstrictor response to inhaled histamine and adenosine 5'-monophosphate in non-atopic asthma. *Thorax* 1987; 42:939-945.
5. Pauwels RA, Van der Straeten ME. An animal model for adenosine-induced bronchoconstriction. *Am Rev Respir Dis* 1987; 136:374-378.
6. Crimi N, Palermo F, Oliveri R, Polosa R, Settinieri I, Mistretta A. Protective effects of inhaled ipratropium bromide on bronchoconstriction induced by adenosine and methacholine in asthma. *Eur Respir J* 1992; 5:560-565.
7. Meijer RJ, Kerstjens HAM, Arends LR, Kauffman HF, Koëter GH, Postma DS. Effects of inhaled fluticasone propionate and oral prednisolone on clinical and inflammatory parameters in patients with asthma. *Thorax* 1999; 54:894-899.
8. Weersink EJ, Douma WR, Postma DS, Koëter GH. Fluticasone propionate, salmeterol xinafoate, and their combination in the treatment of nocturnal asthma. *Am J Respir Crit Care Med* 1997; 155:1241-1246.
9. O'Connor BJ, Ridge SM, Barnes PJ, Fuller RW. Greater effect of inhaled budesonide on adenosine 5'-monophosphate- induced than on sodium-metabisulfite-induced bronchoconstriction in asthma. *Am Rev Respir Dis* 1990; 146:560-564.
10. Benckhuijsen JJ, van den Bos W, van Velzen E, de Bruijn R, Aalbers R. Differences in the effect of allergen avoidance on bronchial hyperresponsiveness as measured by methacholine, adenosine 5'- monophosphate, and exercise in asthmatic children. *Pediatr Pulmonol* 1996; 22:147-153.
11. Alving K, Weitzberg E, Lundberg JM. Increased amount of nitric oxide in exhaled air of asthmatics. *Eur Respir J* 1993; 6:1368-1370.
12. Fahy JV, Liu J, Wong H, Boushey HA. Cellular and biochemical analysis of induced sputum from asthmatic and from healthy subjects. *Am Rev Respir Dis* 1993; 147:1126-1131.
13. Kerstjens HAM, Brand PL, Hughes MD, Robinson NJ, Postma DS, Sluiter HJ, Bleecker ER, Dekhuijzen PN, de Jong PM, Mengelers HJ. A comparison of bronchodilator therapy with or without inhaled corticosteroid therapy for obstructive airways disease. Dutch Chronic Non-Specific Lung Disease Study Group. *N Engl J Med* 1992; 327:1413-1419.
14. Ichinose M, Takahashi T, Sugiura H, Endoh N, Miura M, Mashito Y, Shirato K. Baseline airway hyperresponsiveness and its reversible component: role of airway inflammation and airway calibre. *Eur Respir J* 2000; 15:248-253.
15. Brand PL, Postma DS, Kerstjens HAM, Koëter GH. Relationship of airway hyperresponsiveness to respiratory symptoms and diurnal peak flow variation in patients with obstructive lung disease. The Dutch CNSLD Study Group. *Am Rev Respir Dis* 1991; 143:916-921.
16. Kuwano K, Bosken CH, Pare PD, Bai TR, Wiggs BR, Hogg JC. Small airways dimensions in asthma and in chronic obstructive pulmonary disease. *Am Rev Respir Dis* 1993; 148:1220-1225.
17. Lambert RK, Wiggs BR, Kuwano K, Hogg JC, Pare PD. Functional significance of increased airway smooth muscle in asthma and COPD. *J Appl Physiol* 1993; 74:2771-2781.
18. Cockcroft DW, Ruffin RE, Dolovich J, Hargreave FE. Allergen-induced increase in non-allergic bronchial reactivity. *Clin Allergy* 1997; 7:503-513.
19. Grunberg K, Smits HH, Timmers MC, de Klerk EP, Dolhain RJ, Dick EC, Hiemstra PC, Sterk PJ. Experimental rhinovirus 16 infection. Effects on cell differentials and soluble markers in sputum in asthmatic subjects. *Am J Respir Crit Care Med* 1997; 156:609-616.
20. Schmidt D, Rabe KF. Immune mechanisms of smooth muscle hyperreactivity in asthma. *J Allergy Clin Immunol* 2000; 105:673-682.
21. Sterk PJ, Bel EH. Bronchial hyperresponsiveness: the need for a distinction between hypersensitivity and excessive airway narrowing. *Eur Respir J* 1989; 2:267-274.
22. Jatakanon A, Lim S, Barnes PJ. Changes in sputum eosinophils predict loss of asthma control. *Am J Respir Crit Care Med* 2000; 161:64-72.

23. Polosa R, Ciamarra I, Mangano G, Prosperini G, Pistorio MP, Vancheri C, Crimi N. Bronchial hyperresponsiveness and airway inflammation markers in nonasthmatics with allergic rhinitis. *Eur Respir J* 2000; 15:30-35.
24. Alvarez MJ, Olaguibel JM, Garcia BE, Rodriquez A, Tabar AI, Urbiola E. Airway inflammation in asthma and perennial allergic rhinitis. Relationship with nonspecific bronchial responsiveness and maximal airway narrowing. *Allergy* 2000; 55:355-362.
25. Viksman MY, Liu MC, Bickel CA, Schleimer RP, Bochner BS. Phenotypic analysis of alveolar macrophages and monocytes in allergic airway inflammation. Evidence for activation of alveolar macrophages, but not peripheral blood monocytes, in subjects with allergic rhinitis and asthma. *Am J Respir Crit Care Med* 1997; 155:858-863.
26. Ulrik CS. Peripheral eosinophil counts as a marker of disease activity in intrinsic and extrinsic asthma. *Clin Exp Allergy* 1995; 25:820-827.
27. Frette C, Annesi I, Korobaeff M, Neukirch F, Dore MF, Kauffmann HF. Blood eosinophilia and FEV_1. Cross-sectional and longitudinal analyses. *Am Rev Respir Dis* 1990; 143:987-992.
28. Durham SR, Kay AB. Eosinophils, bronchial hyperreactivity and late-phase asthmatic reactions. *Clin Allergy* 1985; 15:411-418.
29. Taylor KJ, Luksza AR. Peripheral blood eosinophil counts and bronchial responsiveness. *Thorax* 1987; 42:452-456.
30. Dahl R. Monitoring bronchial asthma in the blood. *Allergy* 1993; 48:77-80.
31. Jatakanon A, Lim S, Kharitonov SA, Chung KF, Barnes PJ. Correlation between exhaled nitric oxide, sputum eosinophils, and methacholine responsiveness in patients with mild asthma. *Thorax* 1998; 53:91-95.
32. Lim S, Jatakanon A, Meah S, Oates T, Chung KF, Barnes PJ. Relationship between exhaled nitric oxide and mucosal eosinophilic inflammation in mild to moderately severe asthma. *Thorax* 2000; 55:184-188.

Chapter 4

Corticosteroid-induced improvement in the PC_{20} of adenosine monophosphate is more closely associated with reduction in airway inflammation than improvement in the PC_{20} of methacholine

M. van den Berge[1], H.A.M. Kerstjens[1], R.J. Meijer[1], D.M. de Reus[2], G.H. Koëter[1], H.F. Kauffman[2], D.S. Postma[1]

Departments of Pulmonology[1] and Allergology[2], University Hospital Groningen, Groningen, the Netherlands

American Journal of Respiratory and Critical Care Medicine 2001; 164:1127-1132

ABSTRACT

Introduction

It has been suggested in cross-sectional studies that provocation with adenosine 5'-monophosphate (AMP) more closely reflects the inflammatory process in asthma than provocation with methacholine or histamine.

Aim

To investigate whether the steroid-induced improvement in PC_{20} AMP is more closely associated with the concomitant reduction in airway inflammation than the improvement in PC_{20} methacholine.

Methods

In 120 asthmatic patients, PC_{20} methacholine and PC_{20} AMP as well as sputum induction and measurement of nitric oxide (NO) in exhaled air were performed before and after two weeks treatment with corticosteroids.

Results

Improvement in PC_{20} AMP was solely related to reduction in airway inflammation (i.e. change in the number of sputum eosinophils, lymphocytes, epithelial cells, and concentration of NO in exhaled air). In contrast, improvement in PC_{20} methacholine was related to both reduction in airway inflammation (i.e. change in the number of sputum eosinophils and lymphocytes) and increase in FEV_1 %pred. The total explained variance of the improvement in bronchial hyperresponsiveness was higher for AMP than methacholine (36% versus 22%).

Conclusions

We conclude that PC_{20} AMP is more sensitive to changes in acute airway inflammation than PC_{20} methacholine, further reinforcing the notion that PC_{20} AMP can be a useful tool for monitoring the effects of anti-inflammatory therapy.

INTRODUCTION

Bronchial hyperresponsiveness and airway inflammation are both well-established features of asthma. Thus far, bronchial hyperresponsiveness generally has been measured with methacholine or histamine. These are both direct stimuli, since they exert their effect directly on airway smooth muscle. Another stimulus to measure bronchial hyperresponsiveness is adenosine 5'-monophosphate (AMP). AMP is an indirect stimulus, since it has little effect on airway smooth muscle contraction 'in vitro'.[1] A major action of AMP appears to involve the release of histamine and other preformed mediators from immunologically primed mast cells as AMP-induced bronchoconstriction is associated with a rise of histamine in plasma and bronchoalveolar lavage fluid.[2;3] Moreover, the PC_{20} AMP is inhibited up to 80% by pretreatment with antihistamines.[4] AMP may have some additional actions on neural pathways as well, since the airway response is partially attenuated by atropine and ipratropium bromide.[5;6]

We have recently demonstrated that the PC_{20} AMP is more closely associated with airway inflammation than PC_{20} methacholine.[7] It has been suggested that the PC_{20} AMP is also more sensitive to changes in airway inflammation, since it improves to a greater extent after therapy with corticosteroids than PC_{20} methacholine.[8;9] However, thus far it is unknown whether this greater improvement in PC_{20} AMP is related to reduction in airway inflammation. The aim of our study was to investigate whether steroid-induced improvement in PC_{20} AMP is more closely associated with the concomitant reduction in airway inflammation than improvement in PC_{20} methacholine. To investigate this, inhaled corticosteroids were tapered down in 120 patients with mild to moderately severe asthma.[10] Corticosteroids were subsequently started and the relationship assessed between the corticosteroid-induced improvement in both PC_{20} methacholine and PC_{20} AMP and the concomitant increase in the level of FEV_1 %pred and reduction in airway inflammation. The latter was assessed by exhaled air and the number of inflammatory cells and eosinophil cationic protein (ECP) obtained by sputum induction

PATIENTS AND METHODS

Patients

Patients with a diagnosis of asthma, 18-65 years old, were included if they met the following criteria: PC_{20} methacholine $\leq$ 8 mg/ml, at least one positive skin prick test out of 17 common aero-allergens, reversibility to β_2-agonist $\geq$ 9% of the predicted FEV_1, and ability to expectorate sputum after hypertonic saline inhalation.

Study design

Inhaled corticosteroids were tapered down as described in a previous study.[10] Patients were asked to visit the hospital on two consecutive days when they had discontinued their inhaled corticosteroids completely for three weeks, or earlier, if they experienced (subjective) symptoms of a pending asthma exacerbation for which they felt that treatment with corticosteroids was desirable. Then, patients were randomized to receive treatment for

two weeks with either prednisone (30 mg/day), fluticasone propionate (2000 μg/day), or fluticasone propionate (500μg/day). Before and after two weeks treatment with corticosteroids, measurement of PC_{20} methacholine and PC_{20} AMP as well as sputum induction, and measurement of nitric oxide (NO) in exhaled air were performed as described previously. The study protocol was approved by the local medical ethics committee and all participants gave their written informed consent.

Exhaled nitric oxide

Exhaled nitric oxide (NO) was measured by tidal breathing method using a chemiluminescence analyzer (CLD 700 AL, ECO physics, Switzerland) as described previously.[10]

Airway function

FEV_1 was measured with a calibrated water-sealed spirometer according to standardized guidelines.[10;11] Provocation tests were performed using a 2 minute tidal breathing method, adapted from Cockcroft and coworkers.[12,13] After an initial nebulized saline challenge, subjects inhaled doubling concentrations, ranging from 0.04 to 320 mg/ml for AMP and 0.038 to 19.2 mg/ml for methacholine-bromide, at 5 min intervals.

Sputum induction and sputum processing

Sputum was induced by inhalation of hypertonic saline aerosols as previously described.[10] Fifteen minutes after salbutamol (200 μg) inhalation, hypertonic saline (3%, 4%, and 5%) was nebulized for each concentration during 7 minutes. Whole samples were processed according to the method of Fahy et al with some modifications.[10;14] Eosinophilic cationic protein (ECP) was measured by ImmunoCAP according to instructions provided by Pharmacia (Uppsala, Sweden).

Statistical methods

All calculations of PC_{20} were performed with the base-2 logarithm (log2) since this reflects doubling concentrations and normalizes the distribution. For purposes of analysis, patients already responding to saline were assigned a PC_{20} value half of the lowest concentration applied.[15] Patients not responding to the highest concentration of methacholine or AMP were assigned a value twice the highest concentration applied. Normality of distributions was assessed using Kolmogorov-Smirnov test. If this test resulted in a p-value < 0.05, normalization by logarithmic transformation was attempted. Correlations between variables were calculated by Pearson's rank correlation test in case of normal distribution or by Spearman's rank correlations test otherwise. To determine independent prognostic factors of PC_{20} methacholine and PC_{20} AMP multivariate regression analysis was employed in the stepwise algorithm (SPSS PC+ 10.0); treatment group, use of corticosteroids (yes/no), and number of days corticosteroids were stopped at randomization were entered as covariates.

RESULTS

Patient characteristics

Between September 1995 and July 1997, 120 patients were enrolled into the study (Table 1).

Table 1. Characteristics and cell count data of the study population (n=120).*

	Before therapy	After therapy	Change (Δ)	
Age (yrs)	27 (22 - 38)			
Gender M/F	41/79			
Smoking (%)				
Current	26			
Non-smoker	74			
FEV_1 (%pred.)	81 (67 – 92)	85 (73 – 97)	5.4 (- 0.8 – 12.3)	p < 0.01
PC_{20} meth (mg/ml)	0.54 (0.02 – 7.9)**	1.5 (0.02 – 39.1)**	1.5 (0.6 – 2.5) †	p < 0.01
PC_{20} AMP (mg/ml)	4 (0.02 – 640)**	42.8 (0.02 – 640)**	3.1 (0.4 – 5.6) †	p < 0.01
Exhaled NO (ppb)#	14 (11 – 20)	11 (7 – 15)	- 3.6 (- 7.4 – 0.4)	p < 0.01
Sputum ECP (ng/ml)	76 (33 – 250)	35 (17 – 101)	- 27.8 (- 143 – 0.7)	p < 0.01
Sputum				
Squamous cells (%)	7 (3 – 16)	8 (4 – 19)		
Total cell count (10^3/ml)	519 (230 – 699)	479 (328 – 810)	84 (- 142 – 272)	p = 0.10
Macrophages (10^3/ml)	198 (109 – 369)	275 (142 – 423)	47 (- 41 – 190)	p < 0.01
Lymphocytes (10^3/ml)	9 (4 – 17)	6 (3 – 12)	- 1 (- 8 – 4)	p = 0.06
Neutrophils (10^3/ml)	138 (64 – 298)	165 (80 – 295)	16 (- 85 – 119)	p = 0.17
Eosinophils (10^3/ml)	22 (7 – 60)	3 (0 – 12)	- 11 (- 42 – -1)	p < 0.01
Bronchial epithelial cells (10^3/ml)	10 (4 – 26)	13 (5 – 29)	2 (- 10 – 14)	p = 0.36

*Values are expressed as median with interquartile ranges. **Geometric mean and range between brackets. †Change in PC_{20} AMP and PC_{20} methacholine expressed in doubling concentrations. #The normal range for exhaled nitric oxide in our laboratory is between 6.0 ppb and 10 ppb.

After the start of the treatment, two of the 120 patients were lost from the study (one pregnancy and one loss of study medication). In this study, it was mandatory that patients completely discontinued their inhaled corticosteroids for at least three weeks. During the steroid tapering period, 16 patients returned to the hospital earlier due to symptoms of a pending asthma exacerbation. From these 16 patients, 6 patients still used inhaled corticosteroids at the start of the treatment period (3 patients 400 μg/day budesonide or beclomethasone, 2 patients 250 μg/day fluticasone propionate, and 1 patient 800 μg/day budesonide); the remaining 10 patients had discontinued their inhaled corticosteroids for a median period of 12 days (range 2 - 21 days). It was not possible to perform hyperresponsiveness testing in all patients, due to asthma symptoms and low FEV_1. Thus, we were able to measure the PC_{20} methacholine and the PC_{20} AMP both before and after treatment with corticosteroids in 111 (94%) and 108 (92%) patients respectively. Before treatment with corticosteroids, all patients were, by design, responsive to methacholine ($PC_{20} \leq 8$ mg/ml), and 102 of the 114 (89%) patients were responsive to AMP ($PC_{20} \leq 320$ mg/ml). After treatment with corticosteroids, 97 of the 111 (87%) patients were responsive to methacholine, whereas 78 of the 108 (72%) patients were responsive to AMP.

Effect of steroid therapy

The PC_{20} methacholine and PC_{20} AMP both improved significantly with steroid therapy by 1.5 and 3.1 doubling concentrations respectively (Table 1). Furthermore, the level of FEV_1 %pred increased significantly after therapy with corticosteroids, whereas the number of eosinophils and macrophages in sputum, the concentration of ECP in sputum and the concentration of nitric oxide (NO) in exhaled air all decreased significantly after therapy with corticosteroids.

Monovariate correlations of corticosteroid-induced changes in PC_{20} methacholine and PC_{20} AMP with concomitant changes in clinical and inflammatory parameters

A significant positive correlation was found between the change in PC_{20} methacholine and PC_{20} AMP with the change in the level of FEV_1 %pred ($r = 0.31$, $p < 0.01$ and $r = 0.36$, $p < 0.01$ respectively) (Table 2, Fig. 1).

Table 2. Individual correlations of corticosteroid-induced changes in clinical and inflammatory parameters with improvement in PC_{20} methacholine and in PC_{20} AMP.

	ΔPC_{20} Methacholine†	ΔPC_{20} AMP†
ΔFEV_1 %predicted	**r = 0.31, p = 0.001**	**r = 0.36, p = 0.0001**
Sputum differential		
Δeosinophils† (10^3/ml)	**r = - 0.28, p = 0.004**	**r = - 0.43, p = 0.000005**
Δlymphocytes† (10^3/ml)	**r = 0.27, p = 0.052**	r = 0.15, p = 0.132
Δmacrophages† (10^3/ml)	**r = 0.25, p = 0.01**	r = 0.01, p = 0.901
Δneutrophils† (10^3/ml)	r = 0.14, p = 0.15	r = - 0.08, p = 0.441
Δbronchial epithelial cells† (10^3/ml)	r = 0.05, p = 0.59	r = - 0.14, p = 0.17
Δsputum ECP† (ng/ml)	r = - 0.07, p = 0.483	**r = - 0.24, p = 0.015**
ΔNO exhaled breath (ppb)	r = - 0.18, p = 0.065	**r = - 0.38, p = 0.0001**

†log transformed to normalize the distribution.

There was a significant negative correlation between the change in PC_{20} methacholine and PC_{20} AMP on one hand and the change in the number of sputum eosinophils on the other hand, the correlation being stronger for change in PC_{20} AMP ($r = - 0.28$, $p < 0.01$ and $r = - 0.43$, $p < 0.01$ respectively) (Fig. 2). Furthermore, there were significant negative correlations between changes in PC_{20} AMP and the change in the concentration of ECP in sputum and NO in exhaled air and significant positive correlations between changes in PC_{20} methacholine and the change in the number of sputum lymphocytes and macrophages. Other correlations were not significant.

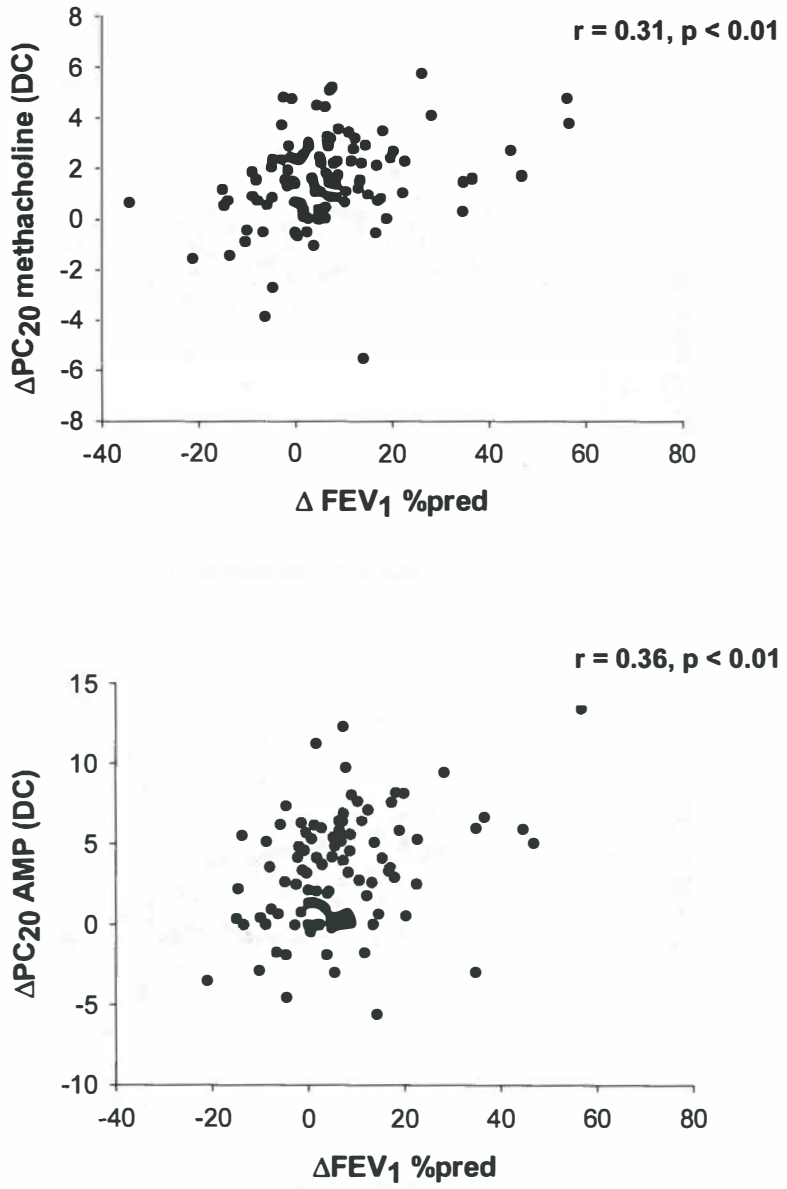


Figure 1. Relationship of improvement in PC_{20} methacholine and in PC_{20} AMP (expressed in doubling concentrations (DC)) to the increase in FEV_1 %predicted.

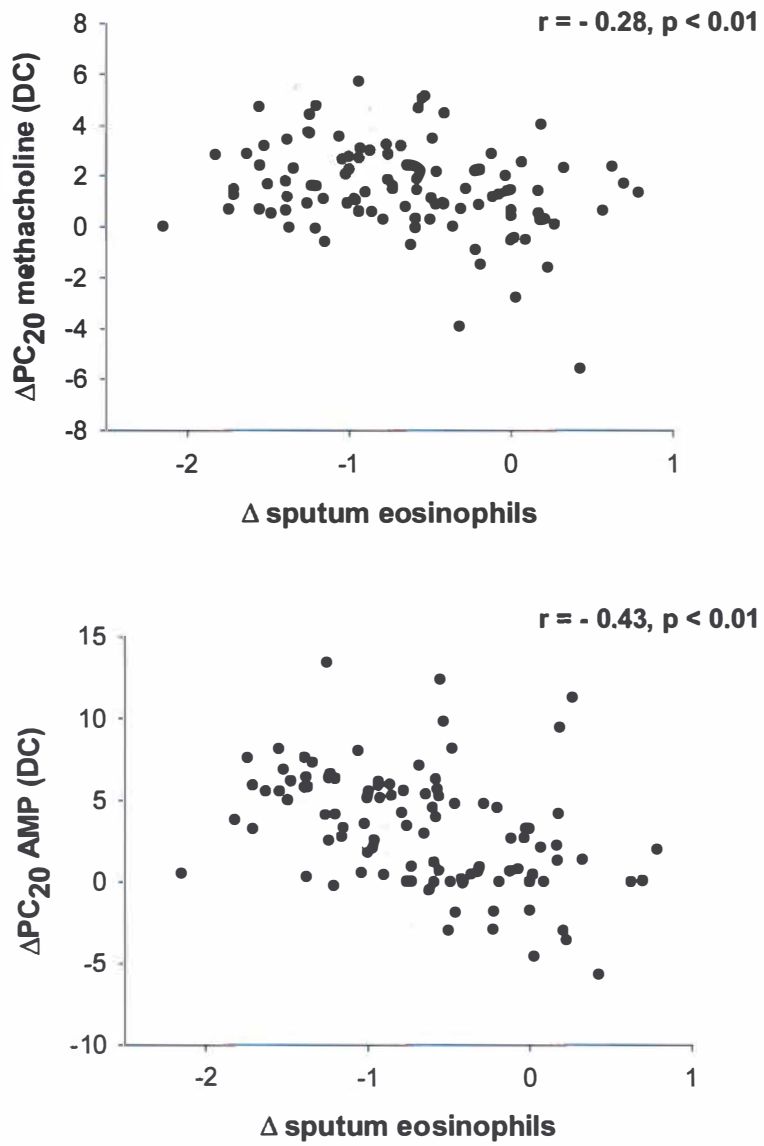


Figure 2. Relationship of improvement in PC_{20} methacholine and in PC_{20} AMP (expressed in doubling concentrations (DC)) to the decrease in the number of sputum eosinophils. The value for the number of sputum eosinophils is log transformed to normalize the distribution.

Multivariate analysis of corticosteroid-induced changes in PC_{20} methacholine and PC_{20} AMP

In a multivariate linear stepwise regression model, both changes in PC_{20} methacholine and PC_{20} AMP were independently negatively correlated with the change in the number of eosinophils and positively with the change in the number of lymphocytes in sputum (Table 3). In addition, the change in the level of FEV_1 %pred was independently and positively correlated with the change in PC_{20} methacholine, but not PC_{20} AMP. The change in the concentration of NO in exhaled air and the change in the number of bronchial epithelial cells in sputum were both independently and negatively correlated with the change in PC_{20} AMP. The total explained variance was larger for changes in PC_{20} AMP than PC_{20} methacholine (total explained variance 36% and 22% respectively). When entering the treatment group, the use of inhaled corticosteroids at randomization (yes/no), and the number of days inhaled corticosteroids were stopped before randomization as covariates, their regression coefficients were not significant.

Table 3. Multiple linear regression models for improvement in PC_{20} methacholine and in PC_{20} AMP.

	ΔPC_{20} methacholine			**ΔPC_{20} AMP**	
	$\beta^{\#}$	Explained variance		$\beta^{\#}$	Explained variance
ΔSputum lymphocytes (10^3/ml)	0.31		ΔSputum eosinophils (10^3/ml)	-0.39	
ΔSputum eosinophils (10^3/ml)	-0.26		ΔSputum lymphocytes (10^3/ml)	0.34	
ΔFEV_1 %pred	0.18		ΔNO exhaled air (ppb)	-0.29	
			ΔSputum bronchial epithelial cells (10^3/ml)	-0.19	
		22%			36%

#Standardized partial correlation coefficient.

DISCUSSION

This study demonstrates that corticosteroid-induced improvement in PC_{20} AMP is more closely associated with the concomitant reduction in airway inflammation than improvement in PC_{20} methacholine. Firstly, improvement in PC_{20} AMP was solely associated with reduction in airway inflammation in the multivariate regression analysis (i.e. the change in the number of sputum eosinophils, lymphocytes, epithelial cells and the concentration of NO in exhaled air), but not with the increase in FEV_1 %pred. In contrast, improvement in PC_{20} methacholine was associated with both reduction in airway inflammation (i.e. the change in the number of sputum lymphocytes and eosinophils) and the increase in FEV_1 %pred. Secondly, the total explained variance of the model estimating the improvement in bronchial hyperresponsiveness by corticosteroids was much higher for AMP than for methacholine (36% versus 22%). The results of this analysis were independent of the treatment group (either prednisone (30 mg/day), fluticasone propionate (2000 µg/day), or fluticasone propionate (500µg/day)).

Both in monovariate and in multivariate regression analyses, improvement in PC_{20} methacholine was associated with the reduction in airway inflammation. It has now been

generally accepted that airway inflammation contributes to the presence and severity of bronchial hyperresponsiveness. However, the direct association between airway inflammation and bronchial hyperresponsiveness has been the subject of much controversy with almost as many negative as positive reports in the literature.[16-20] Therefore, other factors are likely to be involved in the development and maintenance of bronchial hyperresponsiveness.[21] A possible explanation for this can be as follows: bronchial hyperresponsiveness consists of a variable component and a fixed component. The variable component is largely caused by airway inflammation and has been illustrated a lot in allergen exposure tests.[18;22] Activation of different inflammatory cells induces vascular leakage and edema with a concomitant increase in airway wall thickness.[23] In addition, airway smooth muscle cells become activated and together this contributes to an increase in bronchial responsiveness after inhaling a bronchoconstrictor stimulus.[23] The fixed component of bronchial hyperresponsiveness is caused by structural changes in the airway wall, which have been consistently found in asthmatic airways and are generally referred to as 'airway remodeling'. Airway remodeling causes thickening of the airway wall due to the deposition of fibrous proteins, an increase in airway smooth muscle mass due to hypertrophy and hyperplasia, and hypertrophy of mucus secreting glands.[24;25] Furthermore, the contractile force of airway smooth muscle increases due to hypertrophy and hyperplasia.[26] It has indeed been demonstrated that a higher amount of reticular basement membrane thickening, a component of airway remodeling consistently found in asthmatic airways, is associated with more severe bronchial hyperresponsiveness and a lower level of FEV_1.[27-29] It is difficult to dissect the variable and the fixed component in cross-sectional studies, when only one measurement is available. Longitudinal studies are able to dissect this variable component from the fixed component, since by definition it is the variable component that changes over time.

In agreement with this, Ichinose and colleagues recently demonstrated that the variable component of PC_{20} methacholine, i.e. its change after therapy with corticosteroids, is associated with improvement airway inflammation, whereas no association between PC_{20} methacholine and airway inflammation could be found at baseline.[30] The findings of Ichinose and colleagues are compatible with ours. We have extended the observation of Ichinose and colleagues in two ways. Firstly, we have measured bronchial responsiveness to both a direct stimulus (methacholine) and an indirect stimulus (AMP). In monovariate regression analysis, improvement in PC_{20} AMP was associated with a reduction in eosinophils as was PC_{20} methacholine. Additionally, improvement in PC_{20} AMP was associated with a decrease in the concentration of ECP in sputum. Secondly, we have performed a multivariate regression analysis.

Interestingly, multivariate regression analysis revealed that steroid-induced improvement in PC_{20} AMP, acting indirectly via the release of mediators from immunologically primed mast cells, is more closely associated with reduction in airway inflammation than improvement in PC_{20} methacholine. The most likely explanation for this is that an overall decreased inflammatory activity results in a decreased production of cytokines and other mediators that stimulate mast cell chemotaxis, maturation or activation directly or indirectly. Alternatively, the number or activation state of eosinophils may be reduced as a result of a decreased production of inflammatory mediators by mast cells. Another possible explanation may be that corticosteroids exert an effect on the adenosine receptors or the

post receptor mechanisms of mast cells, although thus far such an effect has not been published.

In concordance with others, we found that the PC_{20} AMP improved to a greater extent after treatment with corticosteroids than the PC_{20} methacholine (3.1 versus 1.5 doubling concentrations). This is probably due to a rapidly (within two weeks) achieved reduction in cellular activity.[8;9] It has been shown that the PC_{20} methacholine continues to improve for at least one year with steroid-therapy.[15] Whether this is also the case for PC_{20} AMP is unknown, but the bronchoconstrictor response to another indirect stimulus, exercise, has been shown to reach a plateau after 2 months.[31] In this context it is interesting to speculate that the improvement in PC_{20} methacholine will 'catch up' with improvement in PC_{20} AMP after a longer time period.

Both improvement in PC_{20} methacholine and PC_{20} AMP were associated with the change in the number of sputum lymphocytes. In a recent study of Lemière and coworkers, it was shown that the interobserver repeatability is low for sputum lymphocytes and bronchial epithelial cells.[32] Thus, it is the more remarkable that we were able to find significant differences, since a greater variability of a test will result in a decreased statistical power to detect changes. The positive association between improvement in PC_{20} methacholine and PC_{20} AMP and change in the number of lymphocytes suggests that lymphocytes have a protective effect on bronchial hyperresponsiveness. This was an unexpected finding, since it has been shown that lymphocytes contribute to the inflammatory process in asthma.[33;34] However, thus far the exact meaning of lymphocytes in sputum is not entirely clear. In our study, we did not measure activation markers and subsets of lymphocytes. These measurements should be able to help define the exact role for these inflammatory cells in sputum in acute and chronic asthma more precisely in future studies.

We found that improvement in PC_{20} AMP, but not PC_{20} methacholine was associated with a decrease in the number of bronchial epithelial cells in sputum. Epithelial shedding is an important feature of asthma.[35] It has been demonstrated that epithelial cell clumps (creola bodies) are present in increased numbers in sputum of asthma patients. Furthermore, partial epithelial denudation of the basement membrane is frequently observed in mucosal biopsies derived from asthmatic airways.[36] The decrease in the number of epithelial cells may reflect a (start of) restoration of the barrier function of the airways leading to a decrease in bronchial hyperresponsiveness. However, in our study the decrease in the level of bronchial responsiveness to AMP, but not to methacholine was associated with a decrease in the number of bronchial epithelial cells. Therefore it seems more likely that the decrease in the number of epithelial cells in sputum after therapy with corticosteroids reflects an overall decrease in the cellular activation state in asthma, which is associated with the improvement in PC_{20} AMP, but not in PC_{20} methacholine.

Finally, improvement in PC_{20} AMP, but not PC_{20} methacholine was associated with a decrease in the concentration of NO in exhaled air both in monovariate and in multivariate regression analysis. It has been suggested in a number of studies that measurement of NO in exhaled air may give information on the degree of airway inflammation in asthmatic patients. The concentration of NO in exhaled air is increased in patients with asthma.[37] In addition, the concentration of NO decreases after therapy with corticosteroids and increases after allergen exposure.[38;39] Finally, it has been shown that pro-inflammatory cytokines

increase the expression of inducible NO synthase (iNOS) in cultured human airway epithelial cells. Thus, our finding that improvement in PC_{20} AMP is more closely associated with a decrease in the concentration of NO in exhaled air is compatible with the thesis of improvement in PC_{20} AMP being more closely associated with reduction in airway inflammation.

Sputum induction has been shown to be a reliable and reproducible method to assess the extent of airway inflammation.[40] In general, the percentages of inflammatory cells in sputum are analysed. We have chosen not to present the percentages of inflammatory cells in sputum, since a decrease in the percentage of one cell type is inevitably associated with an increase in another cell type. Therefore this mode of expression will influence the results of steroid-induced changes in sputum cell differential counts. To avoid this adverse effect, we have used absolute cell counts in sputum. We have also analyzed the percentages of inflammatory cells in sputum. In that analysis, we similarly found that improvement in PC_{20} methacholine was associated with both the increase in FEV_1 %pred, and reduction in airway inflammation (i.e. percentage of sputum eosinophils and lymphocytes), while improvement in PC_{20} AMP was more closely related to reduction in inflammation and not with a reduction in FEV_1 %pred. The improvement in PC_{20} AMP was independently associated with the decrease in the concentration of NO in exhaled air, and changes in the percentages of lymphocytes, macrophages, bronchial epithelial cells, and eosinophils in sputum. The current analysis of absolute cell counts shows that the change in percentages of sputum macrophages was not a valid association, but merely due to concomitant changes in the percentages of sputum eosinophils and lymphocytes.

In conclusion, it has been demonstrated in this study that improvement in PC_{20} AMP with steroid treatment is more closely associated with reduction in airway inflammation than improvement in PC_{20} methacholine. This finding suggests that PC_{20} AMP is a more powerful tool to monitor active inflammation in the airway wall.

ACKNOWLEDGEMENTS

This study was supported by a grant from the University of Groningen, the University Hospital of Groningen, and GaxoSmithKline.

We thank Pharmacia Uppsala for generally providing the kits for ECP determination.

We thank B. Dijkhuizen and J. Zonderland for their laboratory assistance.

REFERENCES

1. Finney MJ, Karlsson JA, Persson CG. Effects of bronchoconstrictors and bronchodilators on a novel human small airway preparation. *Br J Pharmacol* 1985; 85:29-36.
2. Phillips GD, Ng WH, Church MK, Holgate ST. The response of plasma histamine to bronchoprovocation with methacholine, adenosine 5'-monophosphate, and allergen in atopic nonasthmatic subjects. *Am Rev Respir Dis* 1990; 141:9-13.
3. Polosa R, Ng WH, Crimi N, Vancheri C, Holgate ST, Church MK, Mistretta A. Release of mast-cell-derived mediators after endobronchial adenosine challenge in asthma. *Am J Respir Crit Care Med* 1995; 151:624-9.
4. Phillips GD, Rafferty P, Beasley R, Holgate ST. Effect of oral terfenadine on the bronchoconstrictor response to inhaled histamine and adenosine 5'-monophosphate in non-atopic asthma. *Thorax* 1987; 42:939-45.
5. Pauwels RA, Van der Straeten ME. An animal model for adenosine-induced bronchoconstriction. *Am Rev Respir Dis* 1987; 136:374-8.
6. Crimi N, Palermo F, Oliveri R, Polosa R, Settinieri I, Mistretta A. Protective effects of inhaled ipratropium bromide on bronchoconstriction induced by adenosine and methacholine in asthma. *Eur Respir J* 1992; 5:560-5.
7. Van Den Berge M, Meijer RJ, Kerstjens HAM, De Reus DM, Koëter GH, Kauffman HF, Postma DS. PC_{20} AMP is more closely associated with airway inflammation in asthma than PC_{20} methacholine. *Am J Respir Crit Care Med* 2001; 163:1546-50.
8. Weersink EJ, Douma RR, Postma DS, Koëter GH. Fluticasone propionate, salmeterol xinafoate, and their combination in the treatment of nocturnal asthma. *Am J Respir Crit Care Med* 1997; 155:1241-6.
9. O'Connor BJ, Ridge SM, Barnes PJ, Fuller RW. Greater effect of inhaled budesonide on adenosine 5'-monophosphate- induced than on sodium-metabisulfite-induced bronchoconstriction in asthma. *Am Rev Respir Dis* 1992; 146:560-4.
10. Meijer RJ, Kerstjens HAM, Arends LR, Kauffman HF, Koëter GH, Postma DS. Effects of inhaled fluticasone propionate and oral prednisolone on clinical and inflammatory parameters in patients with asthma. *Thorax* 1999; 54:894-9.
11. Quanjer PH, Tammeling GJ, Cotes JE, Pedersen OF, Peslin R, Yernault JC. Lung volumes and forced ventilatory flows. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. *Eur Respir J Suppl* 1993; 16:5-40.
12. Cockcroft DW, Killian DN, Mellon JJ, Hargreave FE. Bronchial reactivity to inhaled histamine: a method and clinical survey. *Clin Allergy* 1977; 7:235-43.
13. Oosterhoff Y, Jansen MA, Postma DS, Koëter GH. Airway responsiveness to adenosine 5'-monophosphate in smokers and nonsmokers with atopic asthma. *J Allergy Clin Immunol* 1993; 92:773-6.
14. Fahy JV, Liu J, Wong H, Boushey HA. Cellular and biochemical analysis of induced sputum from asthmatic and from healthy subjects. *Am Rev Respir Dis* 1993; 147:1126-31.
15. Kerstjens HAM, Brand PL, Hughes MD, Robinson NJ, Postma DS, Sluiter HJ, Bleecker ER, Dekhuijzen PN, de Jong PM, Mengelers HJ. A comparison of bronchodilator therapy with or without inhaled corticosteroid therapy for obstructive airways disease. Dutch Chronic Non-Specific Lung Disease Study Group. *N Engl J Med* 1992; 327:1413-9.
16. Sont JK, Han J, van Krieken JM, Evertse CE, Hooijer R, Willems LN, Sterk PJ. Relationship between the inflammatory infiltrate in bronchial biopsy specimens and clinical severity of asthma in patients treated with inhaled steroids. *Thorax* 1996; 51:496-502.
17. Lim S, Jatakanon A, John M, Gilbey T, O'Connor BJ, Chung KF, Barnes PJ. Effect of inhaled budesonide on lung function and airway inflammation. Assessment by various inflammatory markers in mild asthma. *Am J Respir Crit Care Med* 1999; 159:22-30.
18. Aalbers R, De Monchy JG, Kauffman HF, Smith M, Hoekstra Y, Vrugt B, Timens W. Dynamics of eosinophil infiltration in the bronchial mucosa before and after the late asthmatic reaction. *Eur Respir J* 1993; 6:840-7.
19. Alvarez MJ, Olaguibel JM, Garcia BE, Rodriquez A, Tabar AI, Urbiola E. Airway inflammation in asthma and perennial allergic rhinitis. Relationship with nonspecific bronchial responsiveness and maximal airway narrowing. *Allergy* 2000; 55:355-62.
20. Bradley BL, Azzawi M, Jacobson M, Assoufi B, Collins JV, Irani AM, Schwartz LB, Durham SR, Jeffery PK, Kay AB. Eosinophils, T-lymphocytes, mast cells, neutrophils, and macrophages in bronchial biopsy specimens from atopic subjects with asthma: comparison with biopsy specimens from atopic subjects without asthma and normal control subjects and relationship to bronchial hyperresponsiveness. *J Allergy Clin Immunol* 1991; 88:661-74.

21. Crimi E, Spanevello A, Neri M, Ind PW, Rossi GA, Brusasco V. Dissociation between airway inflammation and airway hyperresponsiveness in allergic asthma. *Am J Respir Crit Care Med* 1998; 157:4-9.
22. Gauvreau GM, Lee JM, Watson RM, Irani AM, Schwartz LB, O'Byrne PM. Increased numbers of both airway basophils and mast cells in sputum after allergen inhalation challenge of atopic asthmatics. *Am J Respir Crit Care Med* 2000; 161:1473-8.
23. Busse WW. Inflammation in asthma: the cornerstone of the disease and target of therapy. *J Allergy Clin Immunol* 1998; 102:S17-S22.
24. Busse W, Elias J, Sheppard D, Banks-Schlegel S. Airway remodeling and repair. *Am J Respir Crit Care Med* 1999; 160:1035-42.
25. Fish JE, Peters SP. Airway remodeling and persistent airway obstruction in asthma. *J Allergy Clin Immunol* 1999; 104:509-16.
26. Holgate ST, Davies DE, Lackie PM, Wilson SJ, Puddicombe SM, Lordan JL. Epithelial-mesenchymal interactions in the pathogenesis of asthma. *J Allergy Clin Immunol* 2000; 105:193-204.
27. Hoshino M, Nakamura Y, Sim J, Shimojo J, Isogai S. Bronchial subepithelial fibrosis and expression of matrix metalloproteinase-9 in asthmatic airway inflammation. *J Allergy Clin Immunol* 1998; 102:783-8.
28. Chetta A, Foresi A, Del Donno M, Consigli GF, Bertorelli G, Pesci A, Barbee RA, Olivieri D. Bronchial responsiveness to distilled water and methacholine and its relationship to inflammation and remodeling of the airways in asthma. *Am J Respir Crit Care Med* 1996; 153:910-7.
29. Bousquet J, Lacoste JY, Chanez P, Vic P, Godard P, Michel FB. Bronchial elastic fibers in normal subjects and asthmatic patients. *Am J Respir Crit Care Med* 1996; 153:1648-54.
30. Ichinose M, Takahashi T, Sugiura H, Endoh N, Miura M, Mashito Y, Shirato K. Baseline airway hyperresponsiveness and its reversible component: role of airway inflammation and airway calibre. *Eur Respir J* 2000; 15:248-53.
31. Waalkens HJ, Essen-Zandvliet EE, Gerritsen J, Duiverman EJ, Kerrebijn KF, Knol K. The effect of an inhaled corticosteroid (budesonide) on exercise- induced asthma in children. Dutch CNSLD Study Group. *Eur Respir J* 1993; 6:652-6.
32. Lemiere C, Taha R, Chaboilez S, Hamid Q. Comparison of cellular composition of induced sputum analysed by Wright staining and immunocytochemistry. *J Allergy Clin Immunol* 2001; 108:521-523.
33. Gratziou C, Carroll M, Walls A, Howarth PH, Holgate ST. Early changes in T lymphocytes recovered by bronchoalveolar lavage after local allergen challenge of asthmatic airways. *Am Rev Respir Dis* 1992; 145:1259-64.
34. Kidney JC, Wong AG, Efthimiadis A, Morris MM, Sears MR, Dolovich J, Hargreave FE. Elevated B cells in sputum of asthmatics. Close correlation with eosinophils. *Am J Respir Crit Care Med* 1996; 153:540-4.
35. Beasley R, Roche WR, Roberts JA, Holgate ST. Cellular events in the bronchi in mild asthma and after bronchial provocation. *Am Rev Respir Dis* 1989; 139:806-17.
36. Jeffery PK. Bronchial biopsies and airway inflammation. *Eur Respir J* 1996; 9:1583-7.
37. Alving K, Weitzberg E, Lundberg JM. Increased amount of nitric oxide in exhaled air of asthmatics. *Eur Respir J* 1993; 6:1368-70.
38. Kharitonov SA, Yates DH, Barnes PJ. Inhaled glucocorticoids decrease nitric oxide in exhaled air of asthmatic patients. *Am J Respir Crit Care Med* 1996; 153:454-7.
39. Baraldi E, Carra S, Dario C, Azzolin N, Ongaro R, Marcer G, Zacchello F. Effect of natural grass pollen exposure on exhaled nitric oxide in asthmatic children. *Am J Respir Crit Care Med* 1999; 159:262-6.
40. In 't Veen JCCM, de Gouw HW, Smits HH, Sont JK, Hiemstra PS, Sterk PJ, Bel EH. Repeatability of cellular and soluble markers of inflammation in induced sputum from patients with asthma. *Eur Respir J* 1996; 9:2441-7.

Chapter 5

Provocation with AMP, but not methacholine, induces sputum eosinophilia

M. van den Berge[1], H.A.M. Kerstjens[1], D.M. de Reus[2], G.H. Koëter[1], H.F. Kauffman[2], D.S. Postma[1]

Departments of Pulmonology[1] and Allergology[2], University Hospital Groningen, Groningen, the Netherlands

ABSTRACT

Introduction

Bronchial hyperresponsiveness is usually measured with direct stimuli such as methacholine (MCh) or histamine. Adenosine 5'-monophosphate (AMP), which acts indirectly via the secondary release of mediators, is another stimulus to measure bronchial hyperresponsiveness.

Aim

To investigate whether provocation with inhaled AMP itself initiates an inflammatory response resulting in influx of eosinophils into the airway lumen.

Methods

We have included 21 non-smoking atopic asthmatic subjects (mean FEV_1 101% predicted, mean age 34 years). Each subject performed three sputum inductions on different days, at least seven days apart: one without previous provocation, one hour after PC_{20} methacholine, and one hour after PC_{20} AMP.

Results

After provocation with AMP, but not methacholine, the percentage of sputum eosinophils increased significantly (from 1.9 ± 0.5% to 4.5 ± 1% ($p < 0.01$) and 1.9 ± 0.5% ($p = 0.89$)). No changes in percentages of neutrophils, lymphocytes, macrophages, or bronchial epithelial cells were found.

Conclusion

A provocation test with AMP leads to an increased percentage of sputum eosinophils. This observation can not be explained by a non-specific response of the airways to a vigorous bronchoconstriction, since methacholine had no effect on inflammatory cells.

INTRODUCTION

Bronchial hyperresponsiveness and airway inflammation, and in particular eosinophilic inflammation, are characteristic features of asthma. Bronchial hyperresponsiveness is usually measured with methacholine or histamine. These are both direct stimuli, since they exert their effect directly on airway smooth muscle. Another possible stimulus to measure bronchial hyperresponsiveness is adenosine 5'-monophosphate (AMP). At this time, there is increasing interest in the role of AMP as a bronchoconstrictor stimulus in asthma, since there is suggestive evidence that the PC_{20} AMP better reflects airway inflammation than the PC_{20} methacholine.[1-3]

Several lines of evidence indicate that AMP induces bronchoconstriction via the release of mast cell mediators through activation of A_{2B} receptors. Firstly, adenosine enhances the release of a variety of inflammatory mediators like histamine, prostaglandins, leukotrienes, and IL-8 from human mast cells 'in vitro'.[4,5] Secondly, endobronchial instillation of AMP results in an immediate increase in the levels of both histamine and the (mast cell specific) protease tryptase in bronchoalveolar lavage fluid.[6]

In recent years, there has been a tendency to attach less importance to the role of the mast cell as an effector cell in asthma. However, new findings have led to a resurgence of interest in the mast cell and support a reevaluation of its role. In particular, the discovery that mast cells are a source of Th2 type cytokines suggests that mast cell activation can contribute to orchestration of the asthmatic inflammatory response.[7-9] The aim of this study was to investigate whether inhalation of AMP will initiate an inflammatory response resulting in eosinophilic influx in sputum. If so, it can be speculated that the AMP-induced increase in sputum eosinophils will be less pronounced in subjects who use inhaled corticosteroids, since inhaled corticosteroids have a general inhibitory effect on eosinophil recruitment, migration and chemotaxis.[10]

We have included 21 asthmatic patients. Eleven subjects used inhaled corticosteroids. Each subject performed three sputum inductions and blood collections on different days at least seven days apart: one without previous provocation, one hour after PC_{20} methacholine, and one hour after PC_{20} AMP. The study was designed in a crossover fashion such that each subject served as his or her own control. To exclude the possibility that our observations could be explained by a non-specific response of the airways to a vigorous bronchoconstriction, we used methacholine as a control challenge.[11-14]

PATIENTS AND METHODS

Patients

Twenty-one patients with a diagnosis of asthma, 18-65 years old, were included if they met the following criteria: A doctor's diagnosis of asthma, non-smoking, documented PC_{20} methacholine $\leq$ 8 mg/ml, PC_{20} AMP $<$ 320 mg/ml at the first visit, at least one positive skin prick test out of 18 common aero-allergens, and the ability to produce sputum. All patients were required to have stable asthma for at least one month preceding and during the study.

Long acting beta-agonists were not allowed for 48 hours before each visit. We have included both patients who regularly used inhaled corticosteroids and patients who had not used inhaled corticosteroids for at least six months.

Study design

Patients were asked to attend three visits to the hospital with an interval of one to three weeks. At the first visit sputum induction and blood collection was performed one hour after a PC_{20} AMP (AMP visit). At the second and the third visit sputum induction and blood collection were performed in random order either one hour after a PC_{20} methacholine (methacholine visit) or without previous provocation (control visit). Thus, the study was designed in a crossover fashion such that each subject served as his or her own control. The study protocol was approved by the local medical ethics committee and all participants gave their written informed consent.

Airway function

FEV_1 was measured with a calibrated dry wedge spirometer (Jaeger Masterscope, Hoechberg, Germany) according to standardized guidelines.[15,16] Provocation tests were performed using a 2 minute tidal breathing method, adapted from Cockcroft and coworkers.[17,18] After an initial nebulized saline challenge, subjects inhaled doubling concentrations, ranging from 0.04 to 320 mg/ml for AMP (Sigma, St Louis, USA) and 0.038 to 19.6 mg/ml for methacholine-bromide (Sigma, St Louis, USA), at 5 min intervals.

Sputum induction and sputum processing

Sputum was induced by inhalation of hypertonic saline aerosols as previously described.[16] Fifteen minutes after salbutamol (200 μg) inhalation, hypertonic saline (3%, 4%, and 5%) was nebulized for each concentration during 7 minutes. Whole samples were processed according to the method of Fahy et al with some modifications.[16,19] Samples with contamination of > 80% squamous cells were excluded from analyses. Subjects were excluded if they were unable to produce an evaluable sputum sample at one or more study visits. At least 600 non-squamous cells were counted by one investigator who was blinded to the type of challenge and counts were expressed as percentages.

Biochemical assays

The concentration of eosinophilic cationic protein (ECP) in serum and sputum were measured using a fluoroenzyme assay, the ImmunoCAP ECP (provided by Pharmacia, Uppsala, Sweden). The concentration of interleukin-8 (IL-8) in sputum was measured by ELISA (CLB, Amsterdam).

Statistical methods

All calculations of PC_{20} were performed with the base-2 logarithm (log2) since this reflects doubling concentrations and normalizes the distribution. Normality of distributions was assessed using Kolmogorov-Smirnov test. If this test resulted in a p-value < 0.05, normalization by logarithmic transformation was attempted. All data were analyzed using the Student's t-test for matched pairs in case of normal distribution and the Wilcoxon

signed ranks test otherwise. Values are presented as mean ± standard error of the mean (sem) unless stated otherwise. When analyzing the subgroups of patients with and without inhaled corticosteroids, the Wilcoxon signed ranks test was used.

RESULTS

Patient characteristics

Twenty-one patients were included into the study. Eleven patients used inhaled corticosteroids. Baseline characteristics are presented in table 1.

Table 1. Characteristics and cell count data of the study population.*

	All patients n = 21	**With ICS** n = 11	**Without ICS** n = 10	
Age (yrs)	33 ± 2	35 ± 4	31 ± 7	NS
Gender M/F	7/14	5/6	2/8	NS
Smoking, n				
Current	0	0	0	NS
Never	17	7	10	NS
Dose of inhaled corticosteroids**		1073 ± 187		
FEV_1 (%pred.)	103 ± 3	99 ± 3.5	107 ± 4.8	NS
PC_{20} meth (mg/ml) $^{\tau\#}$	0.8 ± 1.4	1.1 ± 0.6	4.3 ± 1.8	NS
PC_{20} AMP (mg/ml) $^{\tau\#}$	11.1 ± 1.4	8.6 ± 1.6	14.6 ± 1.8	NS
Blood eosinophils (10^9/l)	0.2 ± 0	0.2 ± 0.1	0.2 ± 0.1	NS
Sputum ECP (ng/ml)$^{\tau\#}$	61.6 ± 1.4	67.5 ± 1.7	57.8 ± 1.7	NS
Sputum IL-8 (ng/ml)$^{\tau\#}$	982 ± 1.3	790 ± 1.6	1119± 1.4	NS
Sputum				
Total cell count (10^6 cells) $^{\tau\#}$	2.6 ± 1.3	2.1 ± 1.4	3.3 ± 1.4	NS
Macrophages, %	40.9 ± 6.3	42.3 ± 8.4	39.4 ± 10	NS
Lymphocytes, %	1 ± 0.2	1 ± 0.3	1 ± 0.3	NS
Neutrophils, %	55.7 ± 6.6	54.4 ± 8.7	57.2 ± 10.5	NS
Eosinophils, %	1.9 ± 0.5	1.9 ± 0.6	2 ± 0.7	NS
Bronchial epithelial cells, %	0.5 ± 0.2	0.5 ± 0.2	0.5 ± 0.3	NS

*Values are expressed as mean ± standard error of the mean. **Expressed as μg beclomethasone or equivalent (100 μg beclomethasone. 100μg budesonide = 50 μg fluticasone propionate). #Geometric mean and geometric standard error of the mean. τLog transformed to obtain normal distribution.

No significant differences in baseline characteristics were found between patients with and without the use of inhaled corticosteroids. Concentrations of sputum ECP and IL-8 were measured in 16 patients after a PC_{20} AMP, in 17 patients after a PC_{20} methacholine and in 17 respectively 16 patients at the control visit. Blood cell numbers and differential counts were performed in 21 patients after a PC_{20} AMP and in 19 patients after a PC_{20} methacholine and at the control visit. The concentration of ECP in blood was assessed in all patients. We did not find any differences in baseline FEV_1 %predicted between the AMP visit, the methacholine visit and the control visit (103% ± 2.7%, 103% ± 3%, and 103% ± 3% respectively, $p > 0.05$). Further, we found no difference in the maximum percentage fall

in FEV_1 between an AMP challenge and a methacholine challenge (mean ± sem 27% ± 1.2% versus 27% ± 1.6%, p = 0.76).

Changes in inflammatory parameters in induced sputum and blood one hour after a provocation test with either AMP or methacholine.

For the group as a whole (both patients with and without inhaled corticosteroids), the percentage of sputum eosinophils increased significantly after a PC_{20} AMP, but not after a PC_{20} methacholine (from 1.9 ± 0.5% to 4.5 ± 1%, p < 0.01 and 1.9 ± 0.5% to 1.9 ± 0.5% respectively, p = 0.89) (see table 2 and figure 1). In addition, we found that the percentage

Table 2. Blood and sputum inflammatory markers after a PC_{20} with either AMP or methacholine and a control visit.*

	PC_{20} AMP n = 21	PC_{20} methacholine n = 21	Control n = 21
Sputum			
Total Cell Count, (10^6 cells) τ#	2 ± 0.1	1.2 ± 0.1	2.6 ± 0.1
Eosinophils, %	**4.5 ± 1****	1.9 ± 0.5	1.9 ± 0.5
Lymphocytes, %	1.1 ± 0.3	0.8 ± 0.2	1 ± 0.2
Macrophages, %	34 ± 5.2	36.2 ± 5.3	40.9 ± 6.3
Neutrophils, %	59.4 ± 5.5	60.4 ± 5.5	55.7 ± 6.6
Bronchial epithelial cells, %	0.7 ± 0.2	0.8 ± 0.3	0.5 ± 0.3
ECP (ng /ml)τ#	55 ± 1.3	**34.2 ± 1.3****	61.6 ± 1.4
IL-8 (ng/ml)τ#	1327 ± 1.2	1452 ± 1.3	982 ± 1.3
Blood			
Eosinophils (10^9/l)	0.22 ± 0	**0.20 ± 0****	0.21 ± 0
Neutrophils (10^9/l)	4.3 ± 0.3	3.9 ± 0.3	4.1 ± 0.3
Lymphocytes (10^9/l)	2.0 ± 0.7	1.9 ± 0.2	2 ± 0.2
Monocytes (10^9/l)	0.5 ± 0.1	0.52 ± 0	0.5 ± 0
Basophils (10^9/l)	0.05 ± 0.03	0.04 ± 0.05	0.04 ± 0.01
ECP (ng/ml)	10.1 ± 1	10.5 ± 1.5	10.1 ± 1.2

*Values are expressed as mean ± standard error of the mean. #Geometric mean ± geometric standard error of the mean. τLog transformed to obtain normal distribution. **Signifcantly different compared to control.

of eosinophils increased significantly after a PC_{20} AMP in the subgroup of patients who did not use inhaled corticosteroids (from 2.0 ± 0.73% to 4.2 ± 1.2%, p = 0.04), whereas a non-siginificant trend was found in the subgroup of patients who did use inhaled corticosteroids (from 1.9 ± 0.7% to 4.8 ± 1.4%, p = 0.09) (see figure 1). No difference in the AMP-induced increase in sputum eosinophils was found between patients with and without inhaled corticosteroids (2.8 ± 1.5% versus 2.2 ± 0.9%, p = 0.8). The concentration of sputum ECP and the number of eosinophils in blood decreased significantly after methacholine challenge (p = 0.04 and p = 0.01 respectively), but not after AMP challenge (see table 2). No further changes in percentages of inflammatory cells in sputum and peripheral blood were observed after a provocation test with either AMP or methacholine. In addition, the concentration of IL-8 in sputum and ECP in blood did not significantly.

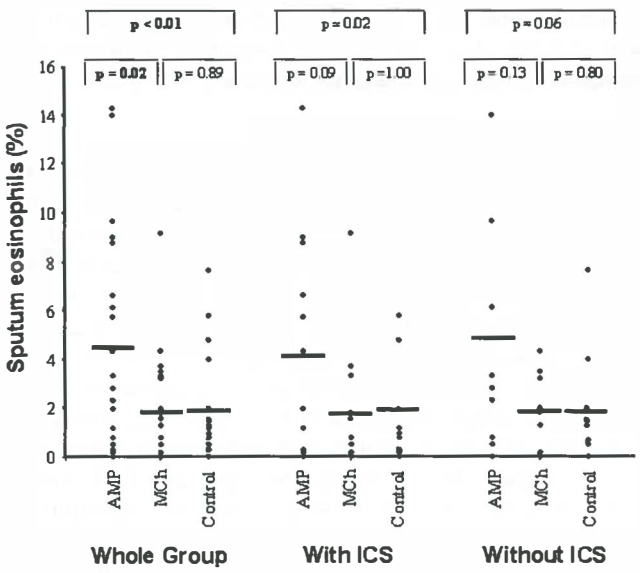


Figure 1. Percentages of sputum eosinophils one hour after provocation with either AMP or methacholine, and at the control visit. Data are presented for all patients (whole group), for patients who used inhaled corticosteroids (with ICS), and for patients who did not use inhaled corticosteroids (without ICS). Horizontal bars represent mean percentage of sputum eosinophils.

change after a provocation test with AMP or methacholine. We have also analyzed our data using total cell counts in sputum instead of percentages of cells. Similarly, the number of eosinophils in sputum tended to increase after a PC_{20} AMP ($p = 0.08$), but not after a PC_{20} methacholine ($p = 0.43$), whereas no changes in numbers of lymphocytes, macrophages, neutrophils or bronchial epithelial cells were observed.

DISCUSSION

This study demonstrates that a provocation test with AMP, but not methacholine, induces an increase in the percentage of sputum eosinophils in patients with asthma. These observations suggest that inhaled AMP has a rapid (within one hour) effect on airway migration of eosinophils in asthmatic subjects.

Our findings of an increase in sputum eosinophils are compatible with the results of Spruntulis and colleagues in an animal model. They observed a significant increase of inflammatory cells (both macrophages and eosinophils) in the bronchoalveolar lavage fluid (BAL) of guinea pigs within one hour after exposure to AMP.[20] We have extended these findings in two ways. Firstly, we have demonstrated that an inflammatory response after inhalation of AMP occurs also in humans. However, we did not observe an increase in macrophages, but a selective increase of eosinophils. This could be due to differences in the airway response to AMP between humans and guinea pigs. The difference in outcome with respect to macrophages may also be due to the use of different compartments of the airways (i.e. BAL and sputum), since sputum has been shown to contain less macrophages than BAL.[21] Secondly, we have also performed sputum induction one hour after a PC_{20}

methacholine as a control challenge and no change in sputum cell differential was observed. This makes it unlikely that the observed increase in sputum eosinophils can be explained by a nonspecific response to a vigorous bronchoconstriction of the airways as has been suggested by Diamant et al.[12]

How can we interpret the findings of our study? Once inhaled, AMP is rapidly converted to adenosine by the ubiquitous enzyme 5'-nucleotidase. Adenosine can activate at least four different adenosine receptors, namely adenosine A_1, A_{2A}, A_{2B}, and A_3 receptors. Activation of A_{2B} receptors present on human mast cells is thought to mediate the bronchoconstrictor response to AMP via the release of a variety of inflammatory mediators.[22] There are various suggestives in the literature that mast cell derived mediators could account for the influx of eosinophils into the airway lumen. Indeed, it has been demonstrated that the proteases tryptase and chymase provide a potent stimulus for eosinophil accumulation following injection in the skin of guinea pigs.[23-25] Further, inhalation of LT_{D4} and LT_{E4}, which are the major lipoxygenase products of mast cells, is associated with increases of eosinophils in sputum and bronchial biopsies.[12,13,26] Finally, the cytokines TNF-alpha and IL-4, which are stored preformed in the granules of mast cells, can facilitate eosinophil recruitment by upregulating adhesion molecules (VCAM-1 and E-selectin) in the endothelial layer of the bronchial vasculature.[27,28] Thus, our finding of an increase in sputum eosinophils after inhalation of AMP suggests that mast cells can play a role in chronic inflammatory conditions in asthma. It could be speculated that the AMP-induced influx in eosinophils might induce a late asthmatic response similar to allergen challenge. However, this was made unlikely by the findings of Phillips and colleagues who could not demonstrate an increase in bronchial hyperresponsiveness to methacholine or a decline in FEV_1 up to 24 hours after AMP challenge.[29]

Another possible explanation for the AMP-induced increase in sputum eosinophils might be that adenosine also exerts an effect on A_1, A_{2A}, and A_3 receptors, which have been identified on neutrophils, eosinophils, and macrophages. The exact consequences of their activation are difficult to predict as various pro- and anti- inflammatory actions have been described: activation of adenosine A_1 receptors promotes chemotaxis of neutrophils and increases adherence of neutrophils to endothelial cells.[30,31] In contrast, activation of adenosine A_{2A} receptors reduces chemotaxis, activation and degranulation of neutrophils and activation of A_3 receptors mediate inhibition of eosinophil chemotaxis when activated.[32,33] The exact consequence of activation of different adenosine receptors has to be sorted out in future studies.

We did not find any changes in percentages of sputum eosinophils, macrophages, lymphocytes, neutrophils or bronchial epithelial cells one hour after provocation with methacholine. This is in agreement with other studies in which no changes in percentages of inflammatory cells in sputum or bronchoalveolar lavage fluid were found within 30 minutes, 1, 4 and 5 hours after provocation with methacholine.[11-14,34] However, we unexpectedly found a decrease in the concentration of sputum ECP and the number of peripheral blood eosinophils. This is in contrast to the findings of Spanevello and colleagues, who did not observe a change in the concentration of sputum ECP after methacholine challenge. A possible explanation for this discrepancy could be that Spanevello and colleagues have evaluated the effect of methacholine challenge on sputum ECP in only 9 patients and this small number may have limited the power to detect

changes. Thus, our findings may indicate an effect of methacholine challenge on the concentration of sputum ECP and the number of peripheral blood eosinophils. This may be relevant for clinical studies in which methacholine challenge, blood collection, and sputum induction are performed at the same visit.

In our study, all subjects performed a PC_{20} AMP followed by sputum induction at the first visit, whereafter they were randomized to either methacholine provocation or baseline sputum induction at the second and the third visit. Thus, a point of criticism to our study design could be that an 'order of measurement bias' can not be excluded. However, we are confident that this study design did not affect our results. Firstly, all study visits were separated by an interval of at least one week, because we felt it likely that the influence of either a provocation test (with AMP or methacholine) or a sputum induction will have disappeared at the subsequent visit. In agreement with this, it has been demonstrated in a recent study that an interval of at least two days between subsequent sputum inductions gives reproducible cell differential counts.[35] In addition, no change in sputum cell differential counts could be observed in previous studies up to four hours after a provocation test with methacholine.[12,36] We are the first to demonstrate that a provocation test with AMP will increase the percentage of eosinophils in sputum and it is unknown whether this increase will have normalized after one week. If not, our study design would have made it even more difficult to detect an AMP-induced increase in sputum eosinophils. Finally, it is unlikely that our results were influenced by seasonal effects, since patients were included into the study throughout the whole year and baseline FEV_1 %predicted values were similar at all visits.

The observed AMP-induced increase in the percentage of sputum eosinophils appeared to be independent of the use of inhaled corticosteroids. This was an unexpected finding, since inhaled corticosteroids have a general inhibitory effect on eosinophil migration and chemotaxis.[37,38] A possible explanation for this observation may be that the severity of asthma (i.e. the level of lung function and the extent and activation of airway inflammation) did not differ at baseline between patients with and without inhaled corticosteroids. It could be speculated that the pre-exisiting increased inflammatory activation state in those patients with inhaled corticosteroids was already counterbalanced by the well known inhibitory effect of inhaled corticosteroids. An alternative explanation may be that AMP induces an increase in sputum eosinophils via a corticosteroid insensitive mechanism.

In conclusion, we have demonstrated that a PC_{20} AMP, but not PC_{20} methacholine increases sputum eosinophilia in asthmatic subjects. Our findings may indicate a role for adenosine and adenosine receptors in asthma, although the exact type of adenosine receptor is yet unresolved. Therefore, it is important to further elucidate the role of adenosine and adenosine receptors in asthma as this may well have consequences for the treatment of asthma.

ACKNOWLEDGEMENTS

This study was supported by an unrestricted educational grant from GlaxoSmithKline.

We thank M. van der Toorn and I. Sloots for their laboratory assistance.

REFERENCES

1. Van Den Berge M, Meijer RJ, Kerstjens HAM, De Reus DM, Koëter GH, Kauffman HF, Postma DS. PC_{20} adenosine 5'-monophosphate is more closely associated with airway inflammation in asthma than PC_{20} methacholine. *Am J Respir Crit Care Med* 2001; 163:1546-50.
2. Van Den Berge M, Kerstjens HAM, Meijer RJ, De Reus DM, Koëter GH, Kauffman HF, Postma DS. Corticosteroid-induced Improvement in the PC(20) of Adenosine Monophosphate Is More Closely Associated with Reduction in Airway Inflammation than Improvement in the PC(20) of Methacholine. *Am J Respir Crit Care Med* 2001; 164:1127-32.
3. Polosa R, Ciamarra I, Mangano G, Prosperini G, Pistorio MP, Vancheri C, Crimi N. Bronchial hyperresponsiveness and airway inflammation markers in nonasthmatics with allergic rhinitis. *Eur Respir J* 2000; 15:30-5.
4. Church MK, Holgate ST, Hughes PJ. Adenosine inhibits and potentiates IgE-dependent histamine release from human basophils by an A_2-receptor mediated mechanism. *Br J Pharmacol* 1983; 80:719-26.
5. Hughes PJ, Holgate ST, Church MK. Adenosine inhibits and potentiates IgE-dependent histamine release from human lung mast cells by an A_2-purinoceptor mediated mechanism. *Biochem Pharmacol* 1984; 33:3847-52.
6. Polosa R, Ng WH, Crimi N, Vancheri C, Holgate ST, Church MK, Mistretta A. Release of mast-cell-derived mediators after endobronchial adenosine challenge in asthma. *Am J Respir Crit Care Med* 1995; 151:624-9.
7. Bradding P, Holgate ST. The mast cell as a source of cytokines in asthma. *Ann N Y Acad Sci* 1996; 796:272-81.
8. Bradding P, Roberts JA, Britten KM, Montefort S, Djukanovic R, Mueller R, Heusser CH, Howarth PH, Holgate ST. Interleukin-4, -5, and -6 and tumor necrosis factor-alpha in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines. *Am J Respir Cell Mol Biol* 1994; 10:471-80.
9. Bradding P, Feather IH, Wilson S, Bardin PG, Heusser CH, Holgate ST, Howarth PH. Immunolocalization of cytokines in the nasal mucosa of normal and perennial rhinitic subjects. The mast cell as a source of IL-4, IL-5, and IL-6 in human allergic mucosal inflammation. *J Immunol* 1993; 151:3853-65.
10. Barnes PJ. Scientific rationale for inhaled combination therapy with long-acting beta2-agonists and corticosteroids. *Eur Respir J* 2002; 19:182-91.
11. Spanevello A, Vignola AM, Bonanno A, Confalonieri M, Crimi E, Brusasco V. Effect of methacholine challenge on cellular composition of sputum induction. *Thorax* 1999; 54:37-9.
12. Diamant Z, Hiltermann JT, van Rensen EL, Callenbach PM, Veselic-Charvat M, van d, V, Sont JK, Sterk PJ. The effect of inhaled leukotriene D4 and methacholine on sputum cell differentials in asthma. *Am J Respir Crit Care Med* 1997; 155:1247-53.
13. Mulder A, Gauvreau GM, Watson RM, O'Byrne PM. Effect of inhaled leukotriene D4 on airway eosinophilia and airway hyperresponsiveness in asthmatic subjects. *Am J Respir Crit Care Med* 1999; 159:1562-7.
14. Kraft M, Bettinger CM, Wenzel SE, Irvin CG, Ackerman SJ, Martin RJ. Methacholine challenge does not affect bronchoalveolar fluid cell number and many indices of cell function in asthma. *Eur Respir J* 1995; 8:1966-71.
15. Quanjer PH, Tammeling GJ, Cotes JE, Pedersen OF, Peslin R, Yernault JC. Lung volumes and forced ventilatory flows. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. *Eur Respir J Suppl* 1993; 16:5-40.
16. Meijer RJ, Kerstjens HAM, Arends LR, Kauffman HF, Koëter GH, Postma DS. Effects of inhaled fluticasone propionate and oral prednisolone on clinical and inflammatory parameters in patients with asthma. *Thorax* 1999; 54:894-9.
17. Cockcroft DW, Killian DN, Mellon JJ, Hargreave FE. Bronchial reactivity to inhaled histamine: a method and clinical survey. *Clin Allergy* 1977; 7:235-43.
18. Oosterhoff Y, Jansen MA, Postma DS, Koëter GH. Airway responsiveness to adenosine 5'-monophosphate in smokers and nonsmokers with atopic asthma. *J Allergy Clin Immunol* 1993; 92:773-6.
19. Fahy JV, Liu J, Wong H, Boushey HA. Cellular and biochemical analysis of induced sputum from asthmatic and from healthy subjects. *Am Rev Respir Dis* 1993; 147:1126-31.
20. Okada S, Kita H, George TJ, Gleich GJ, Leiferman KM. Migration of eosinophils through basement membrane components in vitro: role of matrix metalloproteinase-9. *Am J Respir Cell Mol Biol* 1997; 17:519-28.
21. Pizzichini E, Pizzichini MM, Kidney JC, Efthimiadis A, Hussack P, Popov T, Cox G, Dolovich J, O'Byrne P, Hargreave FE. Induced sputum, bronchoalveolar lavage and blood from mild asthmatics: inflammatory cells, lymphocyte subsets and soluble markers compared. *Eur Respir J* 1998; 11:828-34.

22. Selroos O, Halme M. Effect of a volumatic spacer and mouth rinsing on systemic absorption of inhaled corticosteroids from a metered dose inhaler and dry powder inhaler. *Thorax* 1991; 46:891-4.
23. Walls AF, He S, Teran LM, Buckley MG, Jung KS, Holgate ST, Shute JK, Cairns JA. Granulocyte recruitment by human mast cell tryptase. *Int Arch Allergy Immunol* 1995; 107:372-3.
24. He S, Walls AF. Human mast cell chymase induces the accumulation of neutrophils, eosinophils and other inflammatory cells in vivo. *Br J Pharmacol* 1998; 125:1491-500.
25. Compton SJ, Cairns JA, Holgate ST, Walls AF. Interaction of human mast cell tryptase with endothelial cells to stimulate inflammatory cell recruitment. *Int Arch Allergy Immunol* 1999; 118:204-5.
26. Gauvreau GM, Parameswaran KN, Watson RM, O'Byrne PM. Inhaled leukotriene E(4), but not leukotriene D(4), increased airway inflammatory cells in subjects with atopic asthma. *Am J Respir Crit Care Med* 2001; 164:1495-500.
27. Hirata N, Kohrogi H, Iwagoe H, Goto E, Hamamoto J, Fujii K, Yamaguchi T, Kawano O, Ando M. Allergen exposure induces the expression of endothelial adhesion molecules in passively sensitized human bronchus: time course and the role of cytokines. *Am J Respir Cell Mol Biol* 1998; 18:12-20.
28. Blease K, Seybold J, Adcock IM, Hellewell PG, Burke-Gaffney A. Interleukin-4 and lipopolysaccharide synergize to induce vascular cell adhesion molecule-1 expression in human lung microvascular endothelial cells. *Am J Respir Cell Mol Biol* 1998; 18:620-30.
29. Phillips GD, Holgate ST. Absence of a late-phase response or increase in histamine responsiveness after bronchial provocation with adenosine 5'- monophosphate in atopic and non-atopic asthma. *Clin Sci* 1988; 75:429-36.
30. Cronstein BN, Daguma L, Nichols D, Hutchison AJ, Williams M. The adenosine/neutrophil paradox resolved: human neutrophils possess both A_1 and A_2 receptors that promote chemotaxis and inhibit O_2 generation, respectively. *J Clin Invest* 1990; 85:1150-7.
31. Felsch A, Stocker K, Borchard U. Phorbol ester-stimulated adherence of neutrophils to endothelial cells is reduced by adenosine A_2 receptor agonists. *J Immunol* 1995; 155:333-8.
32. Suzuki H, Takei M, Nakahata T, Fukamachi H. Inhibitory effect of adenosine on degranulation of human cultured mast cells upon cross-linking of Fc epsilon RI. *Biochem Biophys Res Commun* 1998; 242:697-702.
33. Walker BA, Jacobson MA, Knight DA, Salvatore CA, Weir T, Zhou D, Bai TR. Adenosine A_3 receptor expression and function in eosinophils. *Am J Respir Cell Mol Biol* 1997; 16:531-7.
34. Nowak D, Grimminger F, Jorres R, Oldigs M, Rabe KF, Seeger W, Magnussen H. Increased LT_{B4} metabolites and PG_{D2} in BAL fluid after methacholine challenge in asthmatic subjects. *Eur Respir J* 1993; 6:405-12.
35. Purokivi M, Randell J, Hirvonen MR, Tukiainen H. Reproducibility of measurements of exhaled NO, and cell count and cytokine concentrations in induced sputum. *Eur Respir J* 2000; 16:242-6.
36. Spanevello A, Migliori GB, Sharara A, Ballardini L, Bridge P, Pisati P, Neri M, Ind PW. Induced sputum to assess airway inflammation: a study of reproducibility. *Clin Exp Allergy* 1997; 27:1138-44.
37. Miyamasu M, Misaki Y, Izumi S, Takaishi T, Morita Y, Nakamura H, Matsushima K, Kasahara T, Hirai K. Glucocorticoids inhibit chemokine generation by human eosinophils. *J Allergy Clin Immunol* 1998; 101:75-83.
38. Lantero S, Sacco O, Scala C, Morelli MC, Rossi GA. Eosinophil locomotion and the release of IL-3 and IL-5 by allergen- stimulated mononuclear cells are effectively downregulated in vitro by budesonide. *Clin Exp Allergy* 1996; 26:656-64.

Chapter 6

No effect of an inhaled adenosine A_{2A}-agonist on the allergen induced late asthmatic response

A double-blind, placebo- and fluticasone-controlled study

B. Luijk[1], M. van den Berge[2]*, H.A.M. Kerstjens[2], Lindsey Cass[3], Antony Sabin[3], D.S. Postma[2], and J-W.J. Lammers[1]*

Departments of Pulmonology, University Medical Center Utrecht[1] and University Hospital Groningen[2], the Netherlands. GlaxoSmithKline[3], Greenford, United Kingdom.

*Both authors contributed equally.

ABSTRACT

Introduction

Adenosine receptor activation is suggested to play a role in asthmatic airway inflammation. A recent study in rats showed that treatment with an adenosine $_{2A}$ receptor agonist inhibits the late asthmatic response.

Aim

To investigate whether treatment with an inhaled adenosine $_{2A}$ agonist inhibits the late asthmatic response in human subjects with asthma.

Methods

15 non-smoking atopic asthmatics underwent an allergen challenge after one week twice daily treatment with either an inhaled adenosine $_{2A}$ agonist (GW328267X) 25 μg, fluticasone propionate 250 μg, or placebo. Sputum cell counts, EG2+ cells, interleukin-8 levels, and eosinophil cationic protein were assessed after 4 days of treatment and again on day 8 (approximately 24 hours after the last dose of medication and a subsequent inhaled allergen challenge). Exhaled nitric oxide was determined at baseline, and after four and seven days of treatment.

Results

No protective effect of the inhaled adenosine $_{2A}$ agonist (GW328267X) against the late asthmatic response was found either in terms of the fall in forced expiratory volume in one second or in the inflammatory parameters tested. However, inhaled fluticasone propionate significantly attenuated the late asthmatic response with an accompanying anti-inflammatory effect on sputum eosinophils, eosinophilic cationic protein and exhaled NO. Surprisingly, fluticasone propionate also significantly increased sputum neutrophils after allergen challenge.

Conclusions

One week, twice daily treatment with the inhaled adenosine $_{2A}$ agonist (GW328267X) at the dose given does not inhibit the allergen induced late asthmatic response nor the associated inflammatory response.

INTRODUCTION

Asthma is a chronic inflammatory disorder characterized by recurrent episodes of symptoms of wheezing and chest tightness that are associated with variable airway obstruction and increased bronchial hyperresponsiveness.[1] It is now generally accepted that the main goal of asthma treatment is to improve airway wall inflammation as much as possible.[2;3] Thus, according to the present guidelines, inhaled corticosteroids (ICS) are introduced into the treatment of asthma when inhaled bronchodilators alone are inadequate to control signs and symptoms of the disease.[2] ICS are the most powerful anti-inflammatory treatment currently available. However, despite their anti-inflammatory action they may not be fully effective in all asthmatics.[3;4] Furthermore, chronic treatment with a high dose of ICS may cause local side effects (as well as dose dependent long-term side effects) in a subset of patients.[3] Thus, there is a need for an alternative drug to treat asthma.

Adenosine has been put forward to have important anti-inflammatory functions.[5] Adenosine exerts its effects via activation of four different adenosine receptors. The effects of this activation of the different receptors are partially pro- and anti-inflammatory. A_{2A} receptor activation reduces chemotaxis, activation, and degranulation of neutrophils, and suppresses the release of tryptase from mast cells in vitro in humans.[6-8] In rats an A_{2A}-agonist has been shown to effectively inhibit the late asthmatic response (LAR).[9]

We hypothesized that inhalation of an A_{2A}–agonist, which has currently been developed for human use, would have a similar inhibitory effect on the LAR in human subjects with asthma. To investigate this, we performed a double-blind, randomized, placebo and inhaled corticosteroid controlled, 3-way cross-over study in mild atopic asthmatic subjects. We assessed the effects of one week inhaled treatment twice daily with either an A_{2A}-agonist (GW328267X) 25 μg, fluticasone propionate 250 μg, or placebo on the allergen-induced LAR. The LAR was assessed as the decline in FEV_1 as well as the accompanying increase in airway inflammation as demonstrated by the change in cell differentiation and predefined inflammatory mediators in induced sputum and blood. Attenuation of the LAR was the primary end point. Furthermore, the effect of treatment on exhaled nitric oxide (eNO) was investigated.

METHODS

Patients

Fifteen male patients were included if they met the following criteria: a diagnosis of asthma according to the ATS guidelines, 18-55 years old, non-smoking, $FEV_1 \geq 70$ % predicted, PC_{20} histamine ≤ 8 mg/ml, and at least one positive skin prick test for either house dust mite (HDM), cat, or timothee grass.[10] Subjects only entered the study when they demonstrated at screening a LAR defined as a greater than 15% decrease in FEV_1 from baseline between 3 and 8 hours after allergen challenge.[11] The use of inhaled corticosteroids and oral corticosteroids was not allowed for four and eight weeks preceding the study, respectively. In addition, no other medication was allowed with the exception of

inhaled short-acting $ß_2$-agonists if needed. All patients were required to have stable asthma for at least one month preceding and during the study.

Study design

This two-center study was set up as a randomized, double blind, placebo and inhaled corticosteroid controlled, 3-way cross-over study to investigate the effect of treatment with an A_{2A}-agonist (GW328267X) on the LAR. Inhaled doses were administered twice daily for 6 days and once on the morning of day 7 by a four-place Diskhaler™ (GlaxoSmithKline, Stevenage, United Kingdom). Each actuation contained either an A_{2A}-agonist (25 μg GW328267X), fluticasone propionate 250 μg, or placebo (lactose powder). On the morning of day 7, the final dose was given 0.5 hour prior to the start of an allergen challenge. All patients had to demonstrate disease stability prior to commencing the first dose of medication in each treatment period, as defined by a PC_{20} histamine within 1.5 doubling doses from the individual screening values. The treatment periods were separated by two to three weeks. Sputum inductions were performed pre-dose at day 4 and 8, the latter being 24 hours after allergen challenge, and exhaled NO was measured pre-dose on treatment days 1, 4, and 7. Blood pressure, heart rate, electrocardiograms and symptom registrations (diary card) were recorded to monitor the occurrence of side-effects induced by GW328267X at day 1, 4, and 7 prior to and after dosing. Finally, blood samples were obtained on day 1 and 7 of each treatment period prior to the dose and up to six hours after drug inhalation for the assessment of serum GW328267X concentrations. Blood was also collected for serum eosinophil cationic protein (ECP) up to 6 hrs with an additional time point at 24 hrs on day 7. The medical ethics committees of the University Medical Center Utrecht and the University Hospital Groningen approved the study and all patients gave their written informed consent.

Histamine and allergen challenge

We applied the standardized guidelines for histamine and allergen challenge by the two minutes tidal breathing method using a dry wedge spirometer (a mobile bi-directional digital spirometer; Sensorloop, SensorMedics Corporation, Yorba Linda, Ca, USA in center one, and a Jaeger Masterscope, Hoechberg, Germany in center two).[11] The nebuliser with an output of 0.13 ml/min (model 646, Devilbiss Inc., Somerset, PA, USA) was calibrated once a week. Either house dust mite (dermatophagoides pteronyssinus), cat (felis catus), or timothee grass (phleum pratense) allergen (ALK Abelló, Horsholm, Denmark) were used for the allergen inhalation provocation tests depending on the individual sensitivity of the subject as determined by skin-prick testing at screening. During screening the cumulative dose of allergen was determined to achieve > 15% fall in FEV_1 during the early asthmatic response (EAR) and a subsequent LAR of more than 15% from baseline FEV_1. The same cumulative dose was repeated in each treatment period.

Sputum induction and processing

Sputum was induced by inhalation of hypertonic saline aerosols as previously described.[12] Fifteen minutes after salbutamol (200 μg) inhalation, hypertonic saline (4.5%) was nebulized three times during 5 minutes. Whole sputum samples were processed according to the method of Fahy and colleagues with some modifications.[13] Samples with

contamination of > 80% squamous cells were excluded from analyses. EG2 positive cells were counted on the cytospins. One investigator who was blinded to the type of challenge counted at least 600 non-squamous cells and counts were expressed as percentages.

Biochemical assays

The concentration of ECP in sputum fluid phase and blood were measured using a fluoroenzyme assay, the ImmunoCAP ECP (provided by Pharmacia, Uppsala, Sweden). The concentration of interleukin-8 (IL-8) in sputum fluid phase was measured by ELISA (CLB, Amsterdam). Immunohistochemical staining was performed on sputum cytospins by an antibody clone EG2 that binds to human eosinophil cationic protein (Van Kabi Pharmacia Diagnostics, Uppsala, Sweden) to determine the percentage of positive cells.

Exhaled nitric oxide

A calibrated chemiluminescence NO analyzer (center 1: type LR2500 Logan Research Ltd, Rochester, Kent, UK and center 2: Ecophysics, CLD 700 AL, Duernten, Switzerland) was used to measure exhaled nitric oxide (eNO) at an expiratory flow rate of 50 ml/sec according to the ATS standards at the same time of day for all treatment periods and expressed as parts per billion (ppb).[14] The average of three acceptable measurements was recorded. The eNO recordings were always performed prior to the FEV_1 measurements.

Statistical analysis

Patient characteristics are presented as means with standard deviations with exception of the PC_{20} histamine, which was log transformed to normalize the distribution. Comparisons were performed by calculating the differences between each treatment period (GW328267X or fluticasone propionate) and placebo along with the associated 95% confidence interval (CI) between brackets. The EAR (0-2h) and LAR (3-8h) were expressed as the decline in FEV_1 in liters. They were analyzed by applying the weighed mean as derived from the area under the curve (AUC (EAR: 0-2h) and AUC (LAR: 3-8h) post allergen challenge, respectively) and the lowest (minimum) FEV_1 during EAR and LAR using analysis of covariance (ANCOVA); a fixed effects model was employed using effects for period, treatment, and subject, with baseline FEV_1 at day 7 as the covariate. Analyses of differences of sputum inflammatory cells and inflammatory markers in sputum fluid phase (IL-8 and ECP), EG2 positive cells in sputum, serum ECP, and eNO between treatment periods are expressed in terms of the ratio of the least squares geometric means along with the associated 95% CI (GW328267X/placebo and fluticasone propionate/placebo).

RESULTS

Fifteen patients were included in the study and fourteen patients completed the study; baseline characteristics are presented in table 1. One patient complained of shortness of breath on the third day of the week with the active drug (GW328267X). This was graded as mild and lasted 8 days after the final dose and was a reason to discontinue treatment. This patient was excluded at the start of a second treatment period due to instability of the PC_{20} histamine. Other than this isolated event, all treatments were generally well tolerated. The

pharmacokinetic profile showed a rapid increase of GW328267X in the peripheral blood with a peak within 0.5 hours (tmax: 0.42 (0.25, 0.75) on day 1 and 0.25 hours (0.08-1.00) on day 7).

Table 1. Patient Characteristics

	Mean ± SD
n	15
Age (years)	28 ± 12
FEV_1 (% predicted)	95 ± 13
PC_{20} Histamine (mg/ml)*	0.86 (0.7 – 2.3)
Allergen**	10 H; 4 G; 1 C
EAR(0-2 hours: % decline in FEV_1 from baseline)	22 ± 4.1
LAR(3-8 hours: % decline in FEV_1 from baseline)	30 ± 12

*Geometric mean (95% CI) **Allergens used during challenges: H = House Dust Mite, G = grass, C = Cat.

PC_{20} histamine data analyses

Fourteen of the fifteen patients showed a stable PC_{20} histamine as defined within 1.5 doubling concentrations from screening values before the start of treatment on day one of each treatment period. Mean values for PC_{20} histamine were 0.93 mg/ml (0.66, 2.36) before the start of GW328267X treatment, 1.02 mg/ml (0.24, 4.19) before the start of fluticasone propionate treatment, and 0.80 mg/ml (0.57, 2.14) before the start of placebo treatment, respectively.

Effects on FEV_1 during the early and late asthmatic response

After one week treatment with the A_{2A}-agonist (GW328267X) twice daily there was no significant change in the FEV_1 prior to the allergen challenge on day 7 when compared to placebo (FEV_1: 3.87 liter and 3.73 liter, respectively). In contrast, FEV_1 significantly increased after one week treatment with fluticasone propionate when compared to placebo. Treatment with the A_{2A}-agonist (GW328267X) did not protect against the EAR or the LAR (LAR: minimum FEV_1: -0.20 (95% CI: -0.57, 0.16)) liter and weighed mean FEV_1: -0.14 (95%CI: -0.44, 0.15) liter) when compared to placebo, whereas a significant protective effect was demonstrated against both the EAR (minimum FEV_1: 0.86 (95%CI: 0.55, 1.16) liter and weighed mean FEV_1: 0.53 (95%CI: 0.30, 0.76) liter, respectively) and the LAR (minimum FEV_1: 1.19 (0.85, 1.54) liter and weighed mean FEV_1: 0.85 (95%CI: 0.58, 1.13) liter, respectively) after fluticasone propionate treatment when compared to placebo (see figure 1).

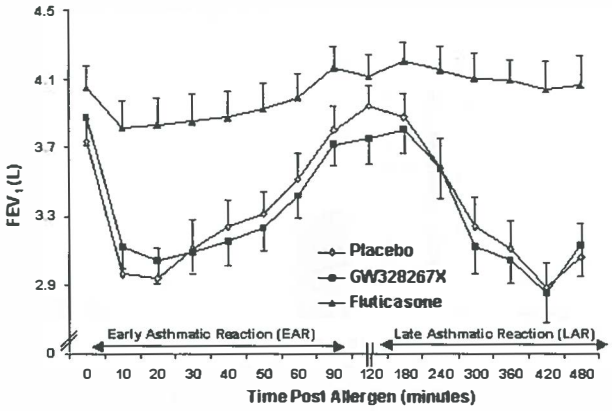


Figure 1. Decrease in mean FEV_1 after allergen challenge on day 7 of treatment with either an A_{2A}-agonist (GW328267X), placebo or fluticasone propionate inhalations twice daily. No significant difference in effects between the A_{2A}-agonist (GW328267X) and placebo was observed, whereas fluticasone propionate induced a significant inhibition of both the early and the late asthmatic response.

Table 2. Sputum cell counts (%) on day 4 during treatment and 24 hours after allergen challenge on day 8.

	DAY 4			DAY 8		
	A_{2A}-agonist	Placebo	FP	A_{2A}-agonist	Placebo	FP
Total cells (x 10^6)	4 (2 – 7)	3 (2 – 7)	5 (3 – 8)	7 (4 – 13)	4 (2 – 8)	5 (3 – 12)
Macrophages, (%)	43 (2 – 57)	46 (31 – 66)	52 (36 – 76)	29 (20 – 43)	29 (18 – 46)	14 (3 – 57)
Lymphocytes, (%)	1 (1 – 3)	2 (1 – 6)	1 (0 – 3)	1 (0 – 4)	1 (1 – 4)	2 (1 – 6)
Neutrophils, (%)	24 (13 – 43)	15 (5 – 44)	14 (4 – 46)	15 (5 – 45)	12 (3 – 52)	**35 (21 – 59)***
Eosinophils, (%)	5 (2 – 14)	5 (2 – 14)	**2 (1 – 6)***	27 (20 – 37)	14 (5 – 43)	**4 (1 – 14)***

Data expressed by geometric means (95%CI) for the A_{2A}-agonist (GW328267X), placebo, and fluticasone propionate). *Statistically different from placebo (see text for treatment difference).

Effect on sputum cell differential counts

The total and cell differential counts are listed in table 2. Treatment with the A_{2A} agonist (GW328267X) did not significantly change sputum total or cell differential counts after four days treatment or 24 hours after allergen challenge (day 8) when compared to placebo. Although, treatment with fluticasone propionate did not significantly decrease the percentage of sputum eosinophils at treatment day four (ratio: 0.39 (95%CI: 0.12, 1.27); figure 2A), there was a significant inhibition of the increase in sputum eosinophils 24 hours after allergen challenge (ratio: 0.29 (95%CI:0.09, 0.96); figure 2B). In addition, the percentage of sputum neutrophils significantly increased 24 hours after allergen challenge (day 8) during fluticasone propionate treatment when compared with placebo (ratio: 3.14 (95%CI:1.17, 8.43); figure 3B); no difference was found 4 days after fluticasone propionate treatment (figure 3A). The other sputum cell differential counts on day 4 and 24 hours after allergen challenge (day 8) with either the A_{2A}-agonist (GW328267X) or fluticasone propionate treatment were not different from placebo treatment.

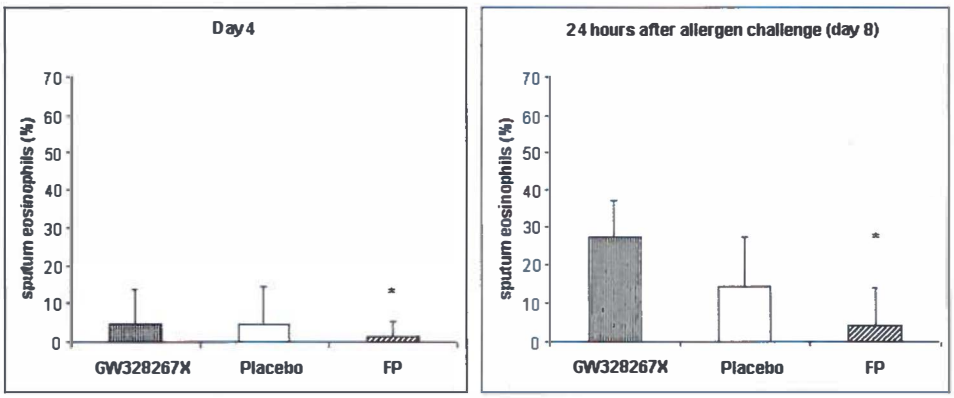


Figure 2. A) Percentage of sputum eosinophils on day 4 during treatment with either the adenosine A_{2A}-agonist (GW328267X), fluticasone propionate, or placebo treatment (geometric means and 95%CI). *Only fluticasone propionate demonstrated a significant difference compared to placebo (for details see text). B) Percentage of sputum eosinophils 24 hours after allergen challenge (day 8) after 8 days treatment with either the A_{2A}-agonist (GW328267X), fluticasone propionate, or placebo (geometric means and CI). Fluticasone propionate treatment significantly inhibited the increase in the percentage of eosinophils after allergen challenge (for details see text), whereas no difference was found between treatment with GW328267X and placebo.

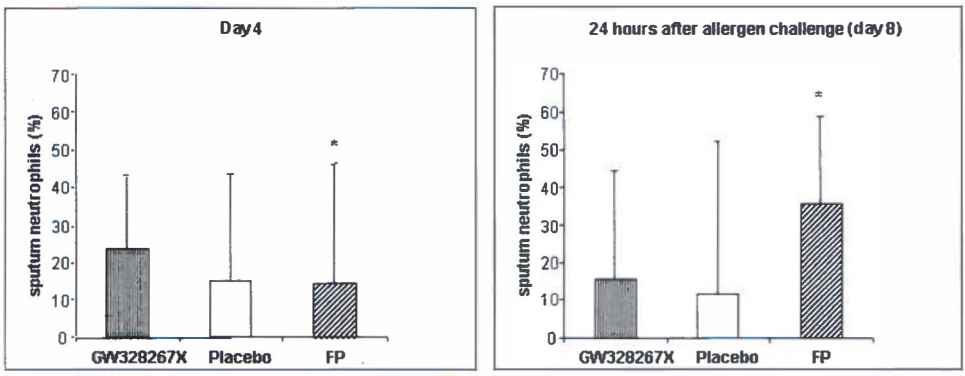


Figure 3. A) Percentage of sputum neutrophils on day 4 during treatment with either the adenosine A_{2A}-agonist (GW328267X), fluticasone propionate, or placebo (geometric means and 95%CI). B) Percentage of sputum neutrophils 24 hours after allergen challenge (day 8) after treatment with either the adenosine A_{2A}-agonist (GW328267X), fluticasone propionate, or placebo (geometric means and CI). Fluticasone propionate treatment significantly increased the percentage of neutrophils compared to placebo (marked by *, for details see text), whereas no difference was found between treatment with GW328267X and placebo.

Effect on inflammatory markers in sputum and blood

Table 3 presents the sputum inflammatory markers. No differences in the ratio of concentrations of sputum ECP, IL-8, and the number of EG2 positive cells were found between treatment with the A_{2A}-agonist and placebo after 4 days treatment or 24 hours after allergen challenge (day 8). In contrast, fluticasone propionate significantly decreased the concentration of sputum ECP when compared to placebo 24 hours after allergen challenge,

whereas this effect was not significant on day 4 (ratios 0.480 (95%CI: 0.02, 1.06) on day 4 and 0.42 (95%CI: 0.19, 0.92) on day 8). No differences in blood ECP concentrations after treatment with the A_{2A}-agonist (GW328267X) or fluticasone propionate were found when compared to placebo.

Effect on eNO measurements

The mean values of the eNO concentrations are shown in figure 5. There were no significant differences in the ratio of eNO concentrations between the A_{2A}-agonist (GW328267X) and placebo after 4 and 7 days treatment. However, there were significant decreases in the concentration of eNO after 4 and 7 days of treatment with fluticasone propionate compared with placebo (ratio on day 4: 0.56 (95%CI: 0.46, 0.67) and a ratio on day 7: 0.41 (95%CI: 0.34, 0.49)).

Table 3. ECP and IL-8 in the sputum fluid phase and EG2 positive cells at day 4 during treatment and 24 hours after allergen challenge on day 8.

	DAY 4			DAY 8		
	A_{2A}-agonist	Placebo	FP	A_{2A}-agonist	Placebo	FP
ECP (ng/L)	65	74	**40**	154	106	**50**
	(33 – 128)	(23 – 238)	**(21 – 76)***	(50 – 469)	(31 – 367)	**(19 – 132)***
IL-8 (pg/L)	3867	5058	3669	4340	3143	4151
	(501 – 5978)	(3249 – 7875)	(2207 – 6099)	(2372 – 7940)	(1134 – 8710)	(2237 – 7702)
EG2+ (%)	3	2	1	7	6	5
	(1 – 14)	(0 – 12)	(0 – 6)	(2 – 33)	(1 – 34)	(1 – 19)

Data expressed by geometric means (95%CI) for the A_{2A}-agonist (GW328267X), placebo, and fluticasone propionate. *Statistically different from placebo (see text for treatment difference).

DISCUSSION

In this study, we found that treatment with an inhaled adenosine receptor $(A)_{2A}$-agonist (GW328267X, 25 μg twice daily) did not protect against the LAR, expressed as the decline in FEV_1 after allergen challenge or the accompanying increase in airway inflammation in asthmatic subjects, whereas fluticasone propionate did so as a positive control.

It has been found that adenosine levels are elevated in bronchoalveolar lavage fluid and in exhaled breath of asthmatics and they increase even further after allergen challenge.[15-17] This raises the possibility that adenosine, once liberated in the asthmatic airways, could itself contribute to the pathophysiology of asthma. Adenosine may be involved in asthmatic airway inflammation by acting on four different adenosine receptors, the A_1, A_{2A}, A_{2B} and A_3 receptors, respectively.[18;19] These adenosine receptors have been put forward as a potential for a new therapeutic approach, since these receptors have been identified on many different inflammatory cells and their activation exert both pro- and anti-inflammatory actions.[19] The A_{2A} receptor is of particular interest, as this receptor possesses anti-inflammatory activities, which may suggest a therapeutic role on the regulation of chronic inflammatory diseases like asthma. A_{2A} receptors are cell surface G-protein coupled receptors that are supposed to downregulate inflammatory responses due the finding of the prolonged and higher pro-inflammatory cytokine levels present in A_{2A} deficient mice.[20]

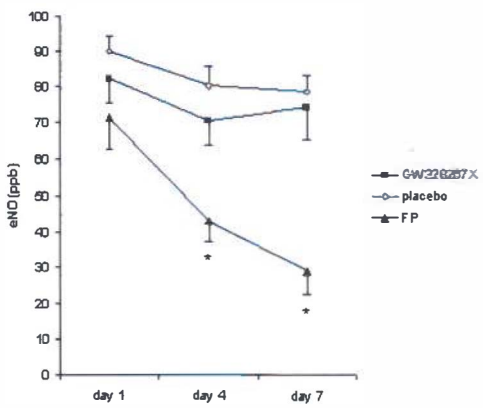


Figure 4. The mean (with standard error of the mean) exhaled nitric oxide concentration in parts per billion (ppb) during treatment with either an inhaled A_{2A}-agonist (GW328267X), fluticasone propionate, or placebo on day 1, day 4, and day 7. A significant decrease was observed after fluticasone propionate treatment on day 4, and on day 7. *significantly different compared to placebo.

It has also been shown in rats that activation of T-lymphocytes, which plays a key role in the recruitment of leukocytes to the lung is suppressed by A_{2A} receptor activation.[21] In addition, adenosine A_{2A} receptor activation results in suppression of FcεR1-induced degranulation of human mast cells and the secretion of IL-12, a pro-inflammatory cytokine, from human monocytes.[8;22] Activation of adenosine A_{2A} receptors also powerfully suppresses T-cell effector functions, neutrophil adherence to the endothelium, upregulation of integrins and activation/degranulation of neutrophils.[6;23-27] Finally, it has recently been reported in an abstract that inhalation of adenosine results in a rapid (within one hour) increase in sputum eosinophils.[28] This array of mechanisms led us to the hypothesize a beneficial anti-inflammatory effect of treatment with an inhaled A_{2A}-agonist in asthma.

Our results are in contrast with a previous study of Fozard and colleagues in rats. They found that treatment with a selective A_{2A}-agonist (CGS21680) significantly inhibited the allergen induced late asthmatic response in sensitized Brown Norway rats.[9] There are several possible explanations for this discrepancy. Firstly, the A_{2A}-agonist we used (GW326287X) was not entirely selective for the A_{2A} receptor, but also exhibits some inhibitory effect on the A_3 receptor. It has recently been shown that inhibition of the A_3 receptor has a pro-inflammatory effect, since the A_3 receptor mediates inhibition of eosinophil chemotaxis when activated and inhibits neutrophil degranulation induced by LPS or TNF-α.[18,27;29] Thus, the inhibitory effect of the drug on A_3 receptors may have counteracted possible beneficial effects of A_{2A} receptor activation. Secondly, it could be argued that the dose of the A_{2A}-agonist we used (25 μg, twice daily by inhalation) has been below the efficacy level either because of a too low dose, or a too short duration of therapy. We deliberately chose a dose of 25 μg GW328267X twice daily, since it was found in mice and in previous studies in healthy non-asthmatic subjects that side effects, i.e. an increase in heart rate and decrease of blood pressure, may occur when higher doses of GW328267X are given (unpublished data). These side effects may be explained by the large tissue distribution of A_{2A} receptors in other cell systems besides inflammatory cells including the heart muscles, coronary arteries, smooth muscle cells, platelets and neuronal cells.[19;23] Finally, the contradiction between the study of Fozard and colleagues in rats and our study

in humans may be due to differences in the distribution and pharmacological profile of the adenosine receptor subtypes between rats and humans. Insufficient work on adenosine receptor distribution in humans has so far been done. Therefore, the relevance of studies with adenosine receptor agonists or antagonists in rats to the clinical human situation is not yet entirely clear.[5]

We have chosen to investigate the effect of the A_{2A}-agonist GW328267X on the inhaled allergen response and we have analyzed 15 patients for this purpose. We feel confident that the sample size was appropriate, since it has been shown that inclusion of 12 patients is already adequate to reliably demonstrate 50% attenuation of the LAR with > 90% power.[30] In agreement with this, we were able to demonstrate a significant attenuation of the LAR after one week treatment with fluticasone propionate 250 μg twice daily. Does a lack of any effect on the allergen-induced late bronchoconstrictory or inflammatory response preclude an effective anti-allergic or anti-inflammatory drug? We are unaware of any such drug making it into clinical medicine, having failed to attenuate the LAR, and we therefore think it is unlikely that A_{2A} receptor agonism by GW328267X at a dose of 25 μg twice daily, will prove to be effective asthma therapy.

Finally, a surprising finding in our study was that the percentage of sputum neutrophils after allergen challenge was higher after one week treatment with inhaled fluticasone propionate when compared to placebo. The increase in neutrophils after fluticasone propionate may be explained by a reduced apoptosis or alternatively by an enhanced recruitment to the airways after allergen challenge as we found no increase in sputum neutrophils after 4 days treatment prior to allergen challenge.[31] In this context, the findings of Jatakanon and colleagues are of interest.[32] They showed that patients with a more severe asthma had higher numbers of sputum neutrophils in their sputum and therefore they suggested that neutrophils contribute to asthma severity. Taken together with our findings, it now becomes tempting to speculate that the observations of Jatakanon and colleagues could be explained by the fact that with more severe asthma higher doses of inhaled corticosteroids were being used.

In conclusion, we have investigated the clinical consequences of activation of the adenosine $_{2A}$ receptor and we could not demonstrate any beneficial effect on the EAR, and the LAR, and the associated increase in airway inflammation. Despite our negative results with the inhaled A_{2A}-agonist GW328267X at a dose of 25 μg twice daily for one week, we feel that the role of adenosine and adenosine receptors in asthma deserves further study. It is well possible that modulation of adenosine receptor subtypes will have therapeutic implications in asthma.

REFERENCES

1. Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM: asthma; from bronchoconstriction to airways inflammation and remodelling. *Am J Respir Crit Care Med* 2000; 161:1720-1745.
2. Global strategy for asthma management and prevention. National Institutes of Health. National Heart, Lung, and Blood Institute, Bethesda, MD. *NHLBI/WHO Workshop Report* 2002.
3. Barnes PJ, Pedersen S, Busse WW. Efficacy and safety of inhaled corticosteroids; new developments. *Am J Respir Crit Care Med* 1998; 157:S1-S53.
4. Chung KF, Godard P, Adelroth E. Difficult/therapy-resistant asthma: the need for an integrated approach to define clinical phenotypes, evaluate risk factors, understand pathophysiology and find novel therapies. ERS Task Force on Difficult/Therapy-Resistant Asthma. European Respiratory Society. *Eur Respir J* 1999; 13:1198-1208.
5. Fozard JR, Hannon JP. Species differences in adenosine receptor-mediated bronchoconstrictor responses. *Clin Exp Allergy* 2000; 30:1213-1220.
6. Cronstein BN, Levin RI, Philips M, Hirschhorn R, Abramson SB, Weissmann G. Neutrophil adherence to endothelium is enhanced via adenosine A_1 receptors and inhibited via adenosine A_2 receptors. *J Immunol* 1992; 148:2201-2206.
7. Visser SS, Theron AJ, Ramafi G, Ker JA, and Anderson R. Apparent involvement of the A_{2A} subtype adenosine receptor in the anti-inflammatory interactions of CGS 21680, Cyclopentyladenosine, and IB-MECA with human neutrophils. *Biochem Pharmacol* 2000; 60:993-999.
8. Suzuki H, Takei M, Nakahata T, Fukamachi H. Inhibitory effect of adenosine on degranulation of human cultured mast cells upon cross-linking of Fc epsilon RI. *Biochem Biophys Res Commun* 1998; 242:697-702.
9. Fozard JR, Ellis KM, Tigani B, Mazzoni L. Effects of CGS 21680, a selective adenosine A_{2A} receptor agonist, on allergic airways inflammation in the rat. *Eur J Pharmacol* 2002; 438:183-188.
10. AmericanThoracic Society. Standards for the diagnosis and care of patients with chronic obstructive disease (COPD) and asthma. *Am Rev Resp Dis* 1987; 136:225-244.
11. Sterk PJ, Fabbri LM, Quanjer PhH. Standardized challenge testing with pharmacological, physical and sensitizing stimuli in adults. *Eur Respir J* 1993; 6:53-83.
12. In 't Veen JCC, De Gouw HWFM, Smits HH. Repeatability of cellular and soluble markers of inflammation in induced sputum from patients with asthma. *Eur Respir J* 1996; 9:2441-2447.
13. Meijer RJ, Kerstjens HA, Arends LR, Kauffman HF, Koëter GH, Postma DS. Effects of inhaled fluticasone propionate and oral prednisolone on clinical and inflammatory parameters in patients with asthma. *Thorax* 1999; 54:894-899.
14. American Thoracic Society. Recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide in adults and children-1999. *Am J Respir Crit Care Med* 1999; 160:2104-2117.
15. Driver AG, Kukoly CA, Ali S, Mustafa SJ. Adenosine in bronchoalveolar lavage fluid in asthma. *Am Rev Respir Dis* 1993; 148:91-97.
16. Mann JS, Holgate ST, Renwick AG, and Cushley MJ. Airway effects of purine nucleosides and nucleotides and release with bronchial provocation in asthma. *J Appl Physiol* 1986; 61:1667-1676.
17. Huszar E, Vass G, Vizi E. Adenosine in exhaled breath condensate in healthy volunteers and in patients with asthma. *Eur Respir J* 2002;20:1393-1398.
18. Van Den Berge M, Kerstjens HAM, and Postma DS. Provocation with adenosine 5'-monophosphate as a marker of inflammation in asthma, allergic rhinitis and chronic obstructive pulmonary disease. *Clin Exp Allergy* 2002; 32:824-830.
19. Polosa R. Adenosine-receptor subtypes: their relevance to adenosine-mediated responses in asthma and chronic obstructive pulmonary disease. *Eur Respir J* 2002; 20:488-496.
20. Ohta A, Sitkovsky M. Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature* 2001; 414:916-920.
21. Huang S, Apasov S, Koshiba M, Sitkovsky M. Role of A_{2A} extracellular adenosine receptor-mediated signaling in adenosine-mediated inhibition of T-cell activation and expansion. *Blood* 1997; 90:1600-1610.
22. Bouma MG, Stad RK, van den Wildenberg FA, Buurman WA. Differential regulatory effects of adenosine on cytokine release by activated human monocytes. *J Immunol* 1994; 153:4159-4168.
23. Gessi S, Varani K, Merighi S, Ongini E, Borea PA: A_{2A} adenosine receptors in human peripheral blood cells. *Br J Pharmacol* 2000; 129:2-11.
24. Thiel M, Chambers JD, Chouker A. Effect of adenosine on the expression of beta(2) integrins and L-selectin of human polymorphonuclear leukocytes in vitro. J Leukoc Biol 1996; 59:671-682.

25. Wollner A, Wollner S, Smith JB: Acting via A_2 receptors, adenosine inhibits the upregulation of Mac-1 (Cd11b/CD18) expression on FMLP-stimulated neutrophils. *Am J Respir Cell Mol Biol* 1993; 9:179-185.
26. Fredholm BB, Zhang Y. Adenosine A_{2A} receptors mediate the inhibitory effect of adenosine on formyl-Met-Leu-Phe-stimulated respiratory burst in neutrophil leucocytes. Naunyn Schmiedebergs *Arch Pharmacol* 1996; 354:262-267.
27. Bouma MG, Jeunhomme TM, Boyle DL. Adenosine inhibits neutrophil degranulation in activated human whole blood: involvement of adenosine A_2 and A_3 receptors. *J Immunol* 1997;158:5400-5408.
28. Van Den Berge M., Kerstjens HAM, de Reus DM, Kauffman HF, Koëter GH, Postma DS: Provocation with AMP increases sputum eosinophils. Am J Respir Crit Care Med 2002; A692(Abstract).
29. Walker BA, Jacobson MA, Knight DA. Adenosine A_3 receptor expression and function in eosinophils. Am J Respir Cell Mol Biol 1997; 16:531-537.
30. Inman MD, Watson R, Cockcroft DW, Wong BJ, Hargreave FE, O'Byrne PM. Reproducibility of allergen-induced early and late asthmatic responses. J Allergy Clin Immunol 1995; 95:1191-1195.
31. Haslett C. Granulocyte apoptosis and its role in the resolution and control of lung inflammation. Am J Respir Crit Care Med 1999; 160:S5-11.
32. Jatakanon A, Uasuf C, Maziak W, Lim S, Chung KF, Barnes PJ. Neutrophilic inflammation in severe persistent ashma. Am J Respir Crit Care Med 1999; 160:1532-1539.

Chapter 7

Altered β_2-adrenergic regulation of T cell activity after allergen challenge in asthma

I.H. Heijink[*†], *M. van den Berge*[†], *E. Vellenga*[†], *D.S. Postma*[‡], *J.G.R. de Monchy*[*], *H.F. Kauffman*[*]

Departments of Allergology[*], Hematology[†] and Pulmonology[‡], University Hospital Groningen, Groningen, the Netherlands

ABSTRACT

Introduction

Allergen-induced airway inflammation in asthma is orchestrated by recruitment of Th2 lymphocytes into lung tissue and production of Th2-like cytokines. We hypothesized that allergen challenge-induced dysfunction of the β_2-adrenergic receptor contributes to enhanced Th2 activity in asthma.

Methods

β_2-adrenergic regulation of cytokine mRNA expression was studied in activated peripheral blood lymphocytes from 7 asthmatic subjects before and 6 hours after allergen challenge. In addition, we studied the effect of fenoterol on T cell chemotaxis and signaling pathways.

Results

After allergen challenge, a complete loss of β_2-adrenergic control over the Th2 cytokines interleukin-4 (IL-4), IL-5 and IL-13, but not the Th1 cytokine IFN-γ, was observed. We further found impaired β_2-adrenergic regulation of T cell migration and signaling pathways, i.e. activation of cAMP responsive element binding protein (CREB) and the mitogen-activated protein kinase (MAPK) pathway. The loss of β_2-adrenergic control was associated with an increased βARK expression, which might be involved in β_2-adrenergic desensitization. In addition, we demonstrate for the first time that T cells exposed to TARC show hyporesponsiveness to fenoterol.

Conclusions

Our results suggest that the allergen-induced loss of β_2-adrenergic control, possibly induced by chemokine release, may play an important role in enhanced T cell activity in asthma.

INTRODUCTION

Atopic asthma is a complex disorder characterized by allergen induced airway inflammation with associated eosinophilia, bronchoconstriction, airway wall edema and bronchial hyperresponsiveness.[1-3] Infiltrating T lymphocytes are thought to orchestrate the immune response associated with asthma by the production of Th2-like cytokines, e.g. interleukin-4 (IL-4), IL-5 and IL-13. Enhanced expression of these cytokines has been observed in bronchoalveolar lavage (BAL) and bronchial biopsies from subjects with asthma.[4-6] After allergen challenge, T cell activity increases, as reflected by further upregulation of Th2 cytokines, enhanced expression of activation markers and migration of T cells into lung tissue.[7-11] Virtually all $IL4^+$ T helper cells that enter the lung upon allergen challenge have been described to express the C-C chemokine receptor CCR4.[12,13] The production of one of the ligands for CCR4, i.e. TARC, is enhanced in bronchial tissue after allergen challenge and may thus be responsible for the attraction of T cells.[12,14] CCR4 is preferentially expressed on Th2 cells, suggesting that primarily Th2-like T cells infiltrate lung tissue upon allergen challenge.[16] Until now, the mechanisms responsible for the enhanced Th2-type activity in asthma are poorly understood.

Hyporesponsiveness of the β_2-adrenergic receptor is a characteristic feature of asthma. The β_2-adrenergic system can suppress inflammatory activity by the activation of the G_s-coupled adenylyl cyclase (AC) system and the subsequent rise in intracellular cAMP.[16,17] T cell-mediated responses can be controlled by cAMP through inhibition of both Th1 and Th2 cytokines, although the ultimate effect of cAMP on Th2 cytokines is dependent on the activation state of the T cell and presence of costimulatory signals.[18,19] The importance of the β_2-adrenergic/AC system in asthma was first demonstrated by the observation that asthma-like phenotypes (e.g. bronchial hyperresponsiveness and eosinophilia) develop in mice and rats when the β-adrenergic system is defect.[20] Furthermore, β_2-adrenergic hyporesponsiveness induced by regular treatment with β_2-agonists increases the sensitivity to allergens in subjects with asthma.[21] Therefore, dysfunction of the β_2-adrenergic receptor may have important implications for airway inflammation.

Allergen induced dysfunction of the β_2-adrenergic system has been observed in peripheral blood lymphocytes within a few hours after allergen challenge.[22,23] This β_2-adrenergic dysfunction may contribute to the increased T cell activity as observed in asthma. Since the infiltration of activated T cells is thought to occur within 24 hours after allergen challenge, we studied β_2-adrenergic regulation of T cell activity 6 hours after allergen challenge. We demonstrate that β_2-adrenergic control over Th2 cytokine production, T cell chemotaxis and signaling pathways is completely abolished after allergen challenge and we discuss the possible underlying mechanism.

MATERIAL AND METHODS

Subjects

Seven patients with a diagnosis of asthma, 18-45 years, were included on the basis of a late obstructive airway response after inhalation of house dust mite (HDM), cat or grass (forced

expiratory volume in one second (FEV_1) during the late asthmatic response (LAR) < 15% from baseline), a provocative concentration of histamine causing 20% fall in FEV_1 from the predicted FEV_1 value (PC_{20}) < 8mg/ml and at least one positive skin prick test (for clinical characteristics see table 1). Patients did not receive oral corticosteroids the preceding two months, nor did they experience respiratory tract infections or acute asthmatic attacks within 4 weeks prior to the start of the study. In addition, no anti-allergic medication was allowed for 4 weeks preceding the study. All patients were required to have stable asthma for at least one month preceding the study. None of the patients were smokers. The Medical Ethics Committee of the University Hospital of Groningen approved the study.

Study design

Blood samples were taken on two subsequent days at similar time points, on the control day and the day of allergen challenge. Allergen provocation took place as described by Aalbers and colleagues.[24] Blood samples were taken six hours after the induction of an early obstructive reaction, as determined by at least a 20% fall in FEV_1 within the first hour after allergen challenge.

Cell cultures, cytokines and reagentia

Peripheral blood mononuclear cells (PBMC's) from asthmatic subjects or healthy donors were isolated by Ficoll-Hypaque (Lymphoprep; Nycomed, Oslo, Norway) density-gradient centrifugation. T cells were isolated by rosetting with 2-aminoethylisothioronium bromide (AET) treated sheep red blood cells (SRBC), as described before.[18] After isolation, the PBMC's or T cells were incubated for 30 min at 37°C in RPMI 1640 (BioWhittaker, Verviers, Belgium) containing 0.5%, 1%, or 5% fetal calf serum (FCS, Hyclone, Logan, UT, USA), supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. T cell-specific stimulation with α-CD3 and α-CD28 (50 µl/min) was used to induce cytokine expression. Fenoterol (Sigma, St. Louis, MO, USA), PGE_2 (Sigma) and sodium fluoride (Sigma) were used in a concentration of 10 µM, 100 nM, and 10 mM, respectively. TARC (R&D systems, ITK diagnostics, Uithoorn, the Netherlands) was used in a concentration of 100 ng/ml.

Measurement of cytokine mRNA expression by LightCycler PCR

3 x 10^6/ml PBMC's were stimulated with α-CD3/α-CD28 during 5 hours, in presence and absence of fenoterol and PGE_2. RNA was isolated using the TRIzol method (GibcoBRL, Burlington, Ontario, Canada) and cDNA was synthesized as described before.[27] Expression of cytokine mRNA was analyzed by quantitative realtime-PCR using the LightCycler system (Roche, Basel, Switzerland) system. Two µl Faststart DNA SybrGreen I mix (Roche), 2mM $MgCl_2$, 0.5 µM of forward and reverse primer and 2 µl cDNA were used. The threshold cycle (Ct) of the α-CD3/α-CD28-stimulated PBMC's in presence of fenoterol or PGE_2 was compared to the Ct generated by a reference sample (the α-CD3/α-CD28 stimulated PBMC). Cytokine gene expression was normalized to expression of the housekeeping gene β_2-microglobulin ($\beta_2\mu G$), with approximately equal amplification efficiency. The ΔCt was calculated as the difference between the Ct values, determined using the equation $2^{-\Delta Ct}$. The specific primer pairs for $\beta_2\mu G$, IL-4, IL-5, IL-13 and IFN-γ

were obtained from Biolegio BV (Malden, the Netherlands). PCR conditions were a denaturation step at 94°C for 10 min followed by 40 cycles of 94°C, 10 s; 58°C, 10s; 72°C, 20 s.

Migration assay

The migration assay was performed using a transwell system with 5 μM pores (Corning Costar Incorporated, New York, MA, USA). 2 x 10^5 T cells were applied to the upper well of the transwell system. Migration was induced by TARC present in the lower well. T cells were allowed to migrate to the lower well for 2 hours, in presence and absence of fenoterol (10 μM) added to the upper well. The upper well was removed and the T cells in the lower well were counted and expressed as percentage of the total amount of T cells added to the upper well.

Immunodetection by western blotting

Phosphorylation of cAMP responsive element binding protein (CREB) and extracellular signal-regulated kinase (ERK) and expression of βARK were analyzed by western blotting. Cells were stimulated with or without fenoterol, PGE_2 or NaF for 60 minutes. In healthy donors, cells were incubated with or without TARC (100 ng/ml) for 6 or 12 hours prior to stimulation. Total cell lysates were prepared, separated by SDS-10% PAGE and transferred to a nitrocellulose membrane. The immunodetection of phospho-CREB, phospho-ERK (New England Biolabs, Hitchin, Herts, UK), pan-ERK and actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was performed according to the manufacturer's guidelines (ECL, Amersham, Buckinghamshire, UK). Relative protein levels were quantified using the gelscan program ImageMaster (Pharmacia, Uppsala, Sweden).

Measurement of IL-5 protein

Freshly isolated T cells (3 x 10^6) were preincubated with or without TARC (100 ng/ml) for 12 hours and stimulated with α-CD3/α-CD28 during 8 hours. Fenoterol (10 μM) was added prior to stimulation. IL-5 protein was measured in cell-free supernatants, using enzyme linked immunosorbent assay (ELISA), performed as previously described by Hoekstra et al.[26]

Statistical analysis

For mRNA measurements, migration of T cells and relative protein levels detected by immunoblotting, statisical analysis was performed using the Wilcoxon's signed rank test for paired observations. Statistical significance of the secretion data was set at $p < 0.05$.

RESULTS

β_2-adrenergic control over Th2 cytokines, but not the Th1 cytokine IFN-γ, is abolished after allergen challenge

We have investigated the β_2-adrenergic control over Th2 cytokines and the Th1 cytokine IFN-γ before and after allergen challenge in 7 asthmatic subjects (baseline characteristics are presented in table 1). The expression of IFN-γ induced by α-CD3/α-CD28 was not significantly different between PBMC's isolated on the control day and after allergen challenge. On the control day, 10 μM fenoterol exerted a strong inhibitory effect on α-CD3/α-CD28-induced mRNA expression of IFN-γ (figure 1, median 43% $\pm$ 14%). Six hours after inhalation of the allergen, the production of IFN-γ was still under strict control of fenoterol (median inhibition from 100% to 40 $\pm$ 10%), the inhibitory effects being not significantly altered.

Table 1. Clinical characteristics of the asthmatic subjects included in our study.

Patient	Gender	Age (yrs)	FEV_1 %pred	FEV_1 (l) t=0	FEV_1 (l) t = 6	PC_{20} histamine mg/ml	allergen
1	m	54	104	3.57	2.62	4.84	grass
2	m	38	73	3.34	2.04	0.22	HDM
3	m	19	105	3.90	2.17	1.84	HDM
4	m	42	83	3.32	2.48	0.37	cat
5	m	21	81	3.80	2.42	0.57	HDM
6	m	27	100	4.85	2.41	1.67	HDM
7	m	18	115	4.02	2.88	1.81	HDM

FEV_1 %pred: predicted value of FEV_1. FEV_1 t = 0: FEV_1 on the control day. FEV_1 t = 6: FEV_1 6 hours after allergen challenge. HDM: house dust mite.

Similarly to IFN-γ, no significant change could be observed in the α-CD3/α-CD28-induced expression of Th2 cytokines after allergen challenge, although the expression of IL-4 (but not IL-5 or IL-13) tended to be upregulated (1.4 $\pm$ 0.5 fold induction, $p = 0.11$). In contrast to IFN-γ, β_2-adrenergic regulation of Th2-type cytokines was significantly altered after allergen challenge. Before allergen challenge, fenoterol modestly but significantly inhibited IL-4, IL-5 and IL-13 mRNA expression (median from 82 $\pm$ 10%, 75 $\pm$ 15% and 70 $\pm$ 24% respectively, figure 1). Allergen inhalation induced a complete loss of β_2-adrenergic control over Th2 cytokine expression. As shown in figure 1, IL-4, IL-5 and IL-13 expressions were no longer significantly inhibited by fenoterol (median 104 $\pm$ 54%, 121 $\pm$ 45% and 120 $\pm$ 32%, respectively) and in some patients, even an upregulatory effect could be observed. In contrast to fenoterol, the effect of PGE_2 on IL-4, IL-5 and IL-13 as well as IFN-γ expression was not significantly altered upon allergen challenge, indicating that the allergen-induced changes in Th2 cytokine regulation are specific for β_2-adrenergic function.

The β_2-adrenergic system controls chemotactic activity in healthy controls and stable asthma

Since little information is available about β_2-adrenergic regulation of T cell migration, this was first studied in freshly isolated T cells from healthy controls. Because of its implications in asthma, we used the chemokine TARC to induce T cell migration. As demonstrated in figure 3A, some spontaneous migration was found (2.4 ± 0.7% of the total number of T cells, n = 6). The amount of T cells migrating to the lower well was significantly enhanced by the addition of TARC, with a maximal response at 100 ng/ml (figure 3A). The addition of fenoterol (10 μM) significantly inhibited the TARC-induced T cell migration, by approximately 40%. The effect of fenoterol on T cell migration was examined in two subjects with asthma, before and 6 hours after allergen challenge. Before challenge, similar results were obtained compared to healthy donors, i.e. TARC induced an approximately two-fold increase in T cell migration, which was inhibited by fenoterol (figure 3B). After allergen challenge, β_2-adrenergic control over T cell migration was abolished, indicating that the loss of β_2-adrenergic control is not specific for Th2 cytokine production.

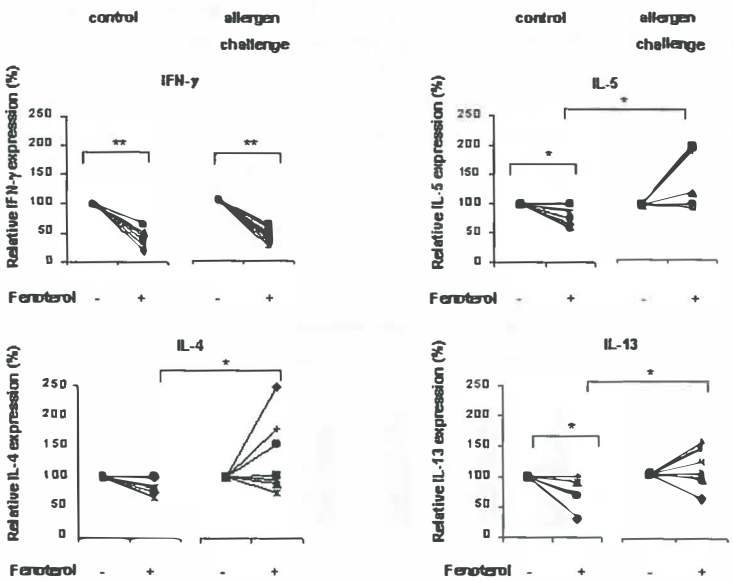


Figure 1. The inhibitory effect of fenoterol on the expression of Th2 (IL-4, IL-5 and IL-13) cytokines, but not Th1 cytokine IFN-γ, is completely abolished after allergen challenge. PBMC's were isolated from 7 asthmatic subjects before (control day) and 6 hours after allergen challenge and stimulated with α-CD3/α-CD28 for 5 hours. Prior to stimulation, 10 μm fenoterol was added. Cytokine mRNA levels are expressed as percentage of the secretion after stimulation with α-CD3/α-CD28 in absence of fenoterol, both before and after allergen challenge. * = $p < 0.05$ and ** = $p < 0.01$ between the indicated values.

Impaired CREB phosphorylation by β_2-adrenergic stimulation after allergen challenge

To investigate whether the reduced sensitivity to fenoterol was caused by the inability to activate the AC/cAMP system, we studied the activation of cAMP downstream effector CREB. In PBMC's derived on the control day, fenoterol induced an increase in the phosphorylation of CREB, which was observed for each patient (figure 4). However, the ability of fenoterol to induce CREB phosphorylation was impaired in PBMC's isolated 6 hours after allergen challenge.In some patients, the induction of CREB phosphorylation was reduced, while in others no induction of CREB phosphorylation could be observed at all. In contrast, both PGE_2 and direct activation of the G protein by NaF induced a strong, significant increase in CREB phosphorylation after challenge (figure 4), suggesting a defect in the β_2-adrenergic receptor.

Impaired regulation of ERK activity by β2-agonist fenoterol after allergen challenge

Th2-like cytokines, which are unlikely regulated by CREB, may be regulated by the interference of cAMP with other pathways. An important pathway in T cell activation that is controlled by cAMP is the Ras/Raf/ERK pathway.[27] cAMP can inhibit this pathway, whereas the desensitized β_2-adrenergic receptor may activate this pathway.[28] We studied the effect of fenoterol on ERK activation before and after allergen challenge. Figure 5 demonstrates an inhibitory effect of fenoterol on ERK phosphorylation in PBMC's isolated on the control day. After allergen challenge, the inhibitory effect was abolished and in some patients ERK activation was even slightly upregulated. This further illustrates altered β_2-adrenergic functionality.

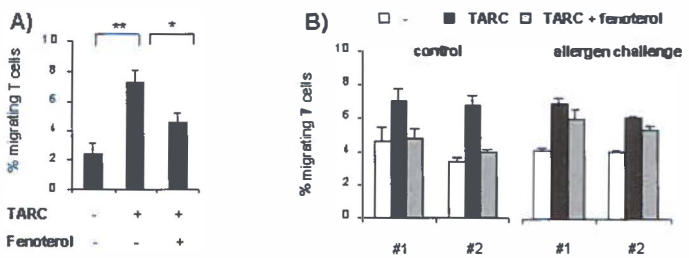


Figure 2. A) The migration of T cells in response to TARC is inhibited by fenoterol. Migration was induced by TARC, in the presence and absence of fenoterol. Migrating T cells are expressed as percentage of the total amount of T cells added to the upper well ($x \pm SEM$, $n = 7$). * = $p < 0.05$ and ** = $p < 0.01$ between the indicated values. B) The inhibitory effect of fenoterol over T cell migration is reduced after allergen challenge. T cells isolated from two asthmatic subjects before (control day) and 6 hours after allergen challenge were used. Migration was induced by TARC, in the presence and absence of fenoterol. T cell migration is expressed as percentage of the total amount of T cells added to the upper well. Experiments were carried out in duplicate and means ± SEM are shown.

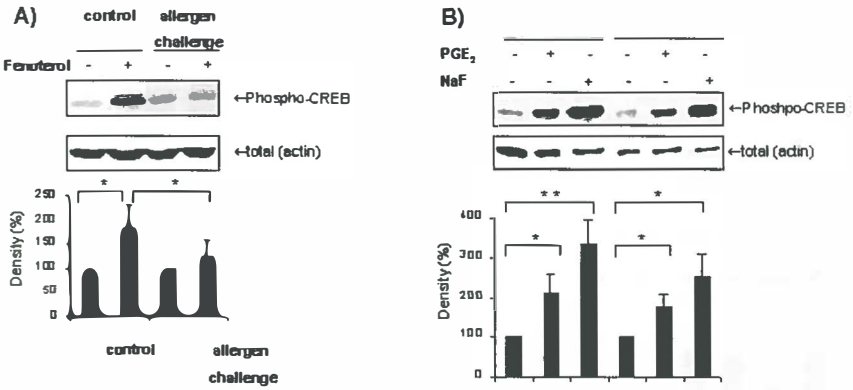


Figure 3. Impaired induction of CREB phosphorylation by fenoterol, but not PGE_2 and NaF, after allergen challenge. PBMC's isolated from asthmatic subjects before (control day) and 6 hours after allergen challenge were stimulated with fenoterol, PGE_2 or NaF for 60 minutes. Total cell lysates were prepared and phosphorylated CREB was detected by western blotting. Phospho-CREB is depicted in the upper panel and total protein levels (actin) are shown in the lower panel (marked by arrows). A representative blot is shown. The phospho-CREB levels were normalized for actin and mean relative phospho-CREB values ± SEM of seven asthmatic subjects are depicted in the corresponding diagram. * = $p < 0.05$ between the indicated values

Upregulation of βARK2 expression after allergen challenge

The G protein-coupled receptor kinase (GRK) βARK is involved in agonist-induced β_2-adrenergic phosphorylation and desensitization, which is enhanced when βARK is overexpressed.[28-32] To study if βARK upregulation could play a role in allergen-induced β_2-adrenergic desensitization, the protein levels of βARK2 (GRK3) were analyzed before and 6 hours after an allergen challenge. Interestingly, the expression of βARK2 significantly increased about a two-fold after allergen challenge (figure 6A, a representative blot and the mean density ± SEM of seven separate experiments in different patients is shown). This enhanced expression of βARK might contribute to the β_2-adrenergic hyporesponsiveness.

Activation of chemokine receptor CCR4 by TARC induces β_2-adrenergic hyporesponsiveness

Because activation of the G_i protein is known to counteract the effects of G_s signaling, we hypothesized that the activation of G_i coupled chemokine receptors might be involved in the reduced β_2-adrenergic responsiveness. We used TARC to induce CCR4 activation and studied the effect on β_2-adrenergic function and βARK expression in T cells obtained from healthy donors. T cells incubated with TARC for 12 hours show stronger βARK2 expression compared to T cells incubated without TARC (figure 7A). In addition, incubation with TARC reduced the capacity of the β_2-agonist fenoterol to induce CREB phosphorylation (figure 7B). This effect was already observed after 6 hours, but more pronounced after 12 hours of preincubation with TARC. Likewise, preincubation with TARC (12 hours) reduced the capacity of fenoterol to control the secretion of the Th2

cytokine IL-5 (figure 7C), while the Th1 cytokine IFN-γ was still strongly inhibited (by approximately 70%, p = 0.01, data not shown). Thus, TARC appears to induce β_2-adrenergic hyporesponsiveness and βARK upregulation, similar to the allergen inhalation in asthma.

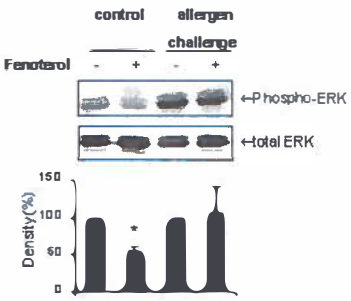


Figure 4. The inhibitory effect of fenoterol on the phoshorylation of ERK-1/2 is abolished after allergen challenge. PBMC's from asthmatic subjects isolated before (control day) and 6 hours after allergen challenge were incubated with fenoterol for 60 minutes. Total cell lysates were prepared and phosphorylated ERK was detected by western blotting. Phospo-ERK is depicted in the upper panel and total protein levels (ERK) are shown in the lower panel (marked by arrows). A representative blot is shown. The phospho-ERK levels were normalized for actin and mean relative phospho-ERK values ± SEM of five asthmatic subjects are depicted in the corresponding diagram. * = $p < 0.05$ between the indicated values.

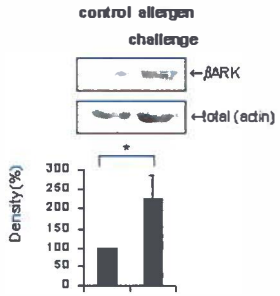


Figure 5. Expression of βARK is enhanced after allergen challenge. Total cell lysates were prepared from PBMC's isolated before (control day) and 6 hours after allergen challenge and expression of βARK (GRK3) was analyzed by western blotting. βARK is depicted in the upper panel and actin is depicted in the lower panel. A representative blot is shown. The βARK levels were normalized for actin and mean relative βARK values ± SEM of 7 asthmatic subjects are depicted in the corresponding diagram.* = $p < 0.05$ between the indicated values.

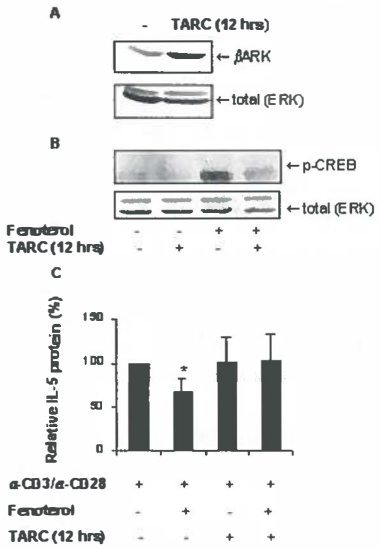


Figure 6. A) Expression of βARK is enhanced in T cells exposed to TARC. T cells isolated from healthy donors were incubated with or without TARC for 12 hours. Total cell lysates were prepared and expression of βARK (GRK3) was analyzed by western blotting. βARK is depicted in the upper panel and total ERK is depicted in the lower panel. A representative blot of three independent experiments is shown. B) Impaired induction of CREB phosphorylation by fenoterol after preincubation with TARC. T cells were isolated from healthy donors, preincubated with or without TARC for 12 hours and stimulated with fenoterol for 60 minutes. Total cell lysates were prepared and phosphorylated CREB was detected by western blotting. Phospho-CREB is depicted in the upper panel and total ERK levels are shown in the lower panel (marked by arrows). A representative blot of five independent experiments is shown. C) The inhibitory effect of fenoterol on IL-5 production is abolished after preincubation with TARC. T cells isolated from healthy donors were preincubated for 12 hours in absence and presence of TARC. T cells were subsequently stimulated with α-CD3/α-CD28 for 8 hours in absence and presence of fenoterol. IL-5 protein levels are expressed as percentage ($x \pm SEM$, $n = 8$) of the secretion after stimulation. * = $p < 0.05$ for the cytokine secretion levels after preincubation with fenoterol compared to the level without (C).

DISCUSSION

An allergen-induced bronchoconstrictive reaction in asthma is associated with enhanced T cell activity.[7-11] Normally, the β_2-adrenergic system is thought to suppress T cell-mediated inflammation. In the present study, we show that allergen inhalation induces a loss of β_2-adrenergic control over the activity of T cells, leading to an enhanced activation profile. While the β_2-agonist fenoterol exerted inhibitory effects on both Th1-like and Th2-like cytokines in stable asthma, Th2 cytokines were no longer inhibited after allergen challenge, whereas the control over Th1 cytokine IFN-γ remained. This may thus favor Th2 activity at the sites of allergen-induced inflammation, where T cell cytokine production is induced. In addition to Th2 cytokines, we observed that the inhibitory effect of fenoterol on T cell chemotaxis is abolished upon allergen challenge in subjects with asthma. Thus, allergen-induced desensitization of the β_2-adrenergic system may facilitate infiltration of T cells into lung tissue. The reduced sensitivity of T cells to fenoterol appears to be a consequence of impaired activation of the cAMP dependent pathway. The associated upregulation in βARK

expression may be one of the mechanisms involved in allergen-induced β_2-adrenergic desensitization. Our findings may explain why airway inflammation in asthma cannot be suppressed by the treatment with β_2-agonists and why β_2-adrenergic desensitization 'in vivo' upon regular use of β_2-agonists renders airways more sensitive to allergen.[21]

Previously, it has been demonstrated that allergen challenge induces impaired AC activity in response to β_2-agonists. This was observed in lymphocytes from subjects with asthma, but not from healthy controls.[22] Furthermore, obstructive reactions caused by histamine inhalation did not impair β_2-adrenergic function, indicating that the release of inflammatory mediators during the allergic reaction is responsible for the β_2-adrenergic dysfunction.[33] There are no indications for enhanced levels of epinephrine after allergen challenge in asthma, suggesting the involvement of heterologous factors.[34]

Our findings concerning CREB indicate that the reduced sensitivity of T cells to the β_2-agonist fenoterol is a consequence of impaired activation of the cAMP-dependent pathway, specific for the β_2-adrenergic receptor. Activation of the G_s protein/AC coupled PGE_2 receptor and direct activation of the G protein by NaF still clearly induced CREB phosphorylation after allergen challenge. Thus coupling of the β_2-adrenergic receptor to the G_s protein/AC system might be reduced. A well-known mechanism involved in the desensitization of the β_2-adrenergic receptor is the activation of the G protein-coupled receptor kinase (GRK) βARK.[29-31] βARK phosphorylates the β_2-adrenergic receptor and enables its binding to β-arrestin, which prevents coupling of the receptor to the G_s protein.[28,30,32] Overexpression of βARK promotes agonist-induced phosphorylation of the β_2-adrenergic receptor.[30] A βARK mediated mechanism may be involved in the allergen-induced β_2-adrenergic dysregulation, since we observed that the that the βARK expression was increased upon allergen challenge. Instead of activation of the AC/cAMP pathway coupling of the β_2-adrenergic receptor to β-arrestin has been described to induce a new set of signaling events, including activation of the MAPK pathway.[28] Indeed, we found that the inhibitory effect of fenoterol on ERK activation, was abrogated after allergen challenge and in some patients we even observed induction of the MAPK/ERK pathway. This might lead to a more activated state of T cells and contribute to the upregulation of Th2 cytokines as observed in some patients.

In a previous study, loss of β_2-adrenergic control over both IFN-γ and IL-5 has been observed 24 hours after allergen challenge.[35] This is possibly the result of further downregulation of β_2-adrenergic function or downregulation in another T cell subset. In contrast to Th2 cytokines, we found that IFN-γ expression remained inhibited by fenoterol after allergen challenge. Since the production of IFN-γ appears to be more sensitive to cAMP than Th2 cytokines, the resting activity of the desensitized β_2-adrenergic AC/cAMP system might still be able to efficiently inhibit IFN-γ. Another explanation for the differential effect on Th1 and Th2 cytokines might be that β_2-adrenergic desensitization predominantly occurs in Th2-like cells. Since virtually all T cells that enter lung tissue after allergen challenge have been demonstrated to express the Th2 specific chemokine receptor CCR4, it is tempting to speculate that particularly these cells loose their negative feedback control. TARC is one of the ligands for CCR4 and the release of TARC into the circulation may be responsible for the attraction of peripheral T cells to the lung after allergen

challenge.[12,14] Most chemokines induce migration through the activation of the receptor-associated G_i protein. The activation of G_i-coupled receptors is known to counteract the effects of G_s activation and may induce βARK-mediated desensitization of G protein-coupled receptors.[36-38]

We found that 'in vitro' preincubation with TARC induced β_2-adrenergic hyporesponsiveness in T cells from healthy donors, with similar alterations as observed in subjects with asthma. Since T cell migration was inhibited by fenoterol, the TARC-induced β_2-adrenergic desensitization might facilitate the migration of $CCR4^+$ cells. Thus, upon the allergen-induced release of TARC, peripheral CCR4+ T cells may change their β_2-adrenergic regulation. As a consequence, the impaired negative feedback control may lead to enhanced activity of the T cells that enter the inflamed tissue.

In summary, our data demonstrate a loss of β_2-adrenergic control in peripheral blood lymphocytes 6 hours after allergen challenge. This impaired β_2-adrenergic function is reflected by a loss of control over TARC-induced T cell migration and Th2 cytokine expression, but not Th1 cytokine expression. The loss of β_2-adrenergic control may promote T cell-mediated inflammation and contribute to enhanced Th2-like activity after allergen challenge in asthma.

ACKNOWLEDGMENTS

This study was supported by grants from the Groningen University Institute for Drug Exploration (GUIDE), "Stichting Astma Bestrijding" (SAB) and Glaxo SmithKline.

We thank the department of Tumor Immunology for providing the α-CD3 and α-CD28 antibodies.

REFERENCES

1. American Thoracic Society. Standards for the diagnosis and care of patients with chronic obstructive pulmonary diseases (COPD) and asthma. *Am Rev Respir Dis* 1987; 136:225-44.
2. Sheth KK, Lemanske RF. Pathogenesis of asthma. *Pediatrician* 1991; 18:257-68.
3. Bradley BL, Azzawi M, Jacobson M, Assoufi B, Collins JV, Irani AM, Schwartz LB, Durham SR, Jeffery PK, Kay AB. Eosinophils, T-lymphocytes, mast cells, neutrophils, and macrophages in bronchial biopsy specimens from atopic subjects with asthma: comparison with biopsy specimens from atopic subjects without asthma and normal control subjects and relationship to bronchial hyperresponsiveness. *J Allergy Clin Immunol* 1991; 88:661-74.
4. Robinson DS, Hamid Q, Ying S, Tsicopoulos J, Barkans J, Bentley CJ, Corrigan CJ, Durham SR, Kay AB. Predominant Th2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med* 1992; 326:298-304.
5. Ying S, Durham SR, Corrigan CJ, Hamid Q, Kay AB. Phenotype of cells expressing mRNA for Th2-type (interleukin 4 and interleukin 5) and Th1-type (interleukin 2 and interferon gamma) cytokines in bronchoalveolar lavage and bronchial biopsies from atopic asthmatic and normal control subjects. *Am J Respir Cell Mol Biol* 1995; 12:477-87.
6. Till S, Durham SR, Dickason R, Huston D, Bungre J, Walker S, Robinson DS, Kay AB, Corrigan CJ. IL-13 production by allergen-stimulated T cells is increased in allergic disease and associated with IL-5 but not IFN-gamma expression. *Immunology* 1997; 91:53-7.
7. Robinson DS, Hamid Q, Bentley A, Ying S, Kay AB, Durham SR. Activation of $CD4^+$ T cells, increased Th2-type cytokine mRNA expression, and eosinophil recruitment in bronchoalveolar lavage after allergen inhalation challenge in patients with atopic asthma. *J Allergy Clin Immunol* 1993; 92:313-24.
8. Huang SK, Xiao HQ, Kleine-Tebbe J, Paciotti G, Marsh DG, Lichtenstein LM, Liu SM. IL-13 expression at the sites of allergen challenge in patients with asthma. *J Immunol* 1995; 55:2688-94.
9. Till SJ, Durham SR, Rajakulasingam K, Humbert M, Huston D, Dickason R. Allergen-induced proliferation and interleukin-5 production by bronchoalveolar lavage and blood T cells after segmental allergen challenge. *Am J Respir Crit Care Med* 1998;158:404-11.
10. Lara-Marquez ML, Moan MJ, Cartwright S, Listman J, Israel E, Perkins EL, Christiani DC, Finn PW. Atopic asthma: differential activation phenotypes among memory T helper cells. *Clin Exp Allergy* 2001; 31:1232-41.
11. Borgonovo B, Casorati G, Frittoli E, Gaffi D, Crimi E, Burastero SE. Recruitment of circulating allergen-specific T lymphocytes to the lung on allergen challenge in asthma. *J Allergy Clin Immunol* 1997; 100:669-78.
12. Panina-Bordignon P, Papi A, Mariani M, Di Lucia P, Casoni G, Bellettato C, Buonsanti C, Miotto D, Mapp C, Villa A, Arrigoni G, Fabbri LM, Sinigaglia F. The C-C chemokine receptors CCR4 and CCR8 identify airway T cells of allergen-challenged atopic asthmatics. *J Clin Invest* 2001; 107:1357-64.
13. Nouri-Aria KT, Wilson D, Francis JN, Jopling LA, Jacobson MR, Hodge MR, Andrew DP, Till SJ, Varga EM, Williams TJ, Pease JE, Lloyd CM, Sabroe I, Durham SR. CCR4 in human allergen-induced late responses in the skin and lung. *Eur J Immunol* 2002; 32:1933-8.
14. Berin MC, Eckmann L, Broide DH, Kagnoff MF. Regulated production of the T helper 2-type T-cell chemoattractant TARC by human bronchial epithelial cells in vitro and in human lung xenografts. *Am J Respir Cell Mol Biol* 2001; 24:382-9.
15. Bonecchi R, Bianchi G, Bordignon PP, D'Ambrosio D, Lang R, Borsatti A, Sozzani S, Allavena P, Gray PA, Mantovani A, Sinigaglia F. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J Exp Med* 1998; 187:129-34.
16. Gilman AG. G proteins:transducers of receptor-generated signals. *Annu Rev Biochem* 1987; 56:615-49.
17. Barnes PJ. Effect of beta-agonists on inflammatory cells. *J Allergy Clin Immunol* 1994; 104:S10.
18. Borger P, Vellenga E, Gringhuis SI, Timmerman AB, Lummen A, Postma DS, Kauffman HF. Prostaglandin E_2 differently modulates interleukin-5 gene expression in activated human T lymphocytes depending on the costimulatory signal. *J Allergy Clin Immunol* 1998; 101:231-36.
19. Hilkens CM, Vermeulen UMH, Van Neerven RJJ, Snijdewint FJM, Wierenga EA, Kapsenberg ML. Differental modulation of T helper type I (Th1) and T helper type 2 (Th2) cytokine secretion by prostaglandin E_2 critically depends on Interleukin-2. *Eur J Immunol* 1995; 25:59-64.
20. Szentivanyi A. The beta-adrenergic theory of the atopic abnormality in bronchial asthma. *J Allergy* 1968; 42:203.
21. Barnes PJ. Beta-adrenergic receptors and their regulation. *Am J Respir Crit Care Med* 1995; 152:838-60.

22. Meurs H, Koëter GH, de Vries K, Kauffinan HF. The beta-adrenergic system and allergic bronchial asthma: changes in lymphocyte beta-adrenergic receptor number and adenylate cyclase activity after an allergen-induced asthmatic attack. *J Allergy Clin Immunol* 1982; 70:272-80.
23. Dooper MW, Timmermans A, Aalbers R, De Monchy JGR, Kauffinan HF. Desensitization of the adenylyl cyclase system in peripheral blood mononuclear cells from patients with asthma three hours after allergen challenge. *J Allergy Clin Immunol* 1993; 92:559-6.
24. Heijink IH, Vellenga E, Borger P, Postma DS, Monchy JGR, Kauffman HF. Polarized Th1 and Th2 cells are less responsive to negative feedback by receptors coupled to the AC/cAMP system compared to freshly isolated T cells. *Br J Pharmacol* 2003; 138:1441-50.
25. Aalbers R, Kauffinan HF, Groen H, Koëter GH, de Monchy JGR. The effect of nedocromil sodium on the early and late reaction and allergen-induced bronchial hyperresponsiveness. *J Allergy Clin Immunol* 1991; 87:993-1001.
26. Hoekstra MO, Hoekstra Y, De Reus DM, Rutgers B, Gerritsen J, Kauffman HF. Interleukin-4, interferon-gamma and interleukin-5 in peripheral blood of children with moderate atopic asthma. *Clin Exp Allergy* 1997; 27:1254-60.
27. Tamir A, Granot Y, Isakov N. Inhibition of T lymphocyte activation by cAMP is associated with down-regulation of two parallel mitogen-activated protein kinase pathways, the extracellular signal-related kinase and c-Jun N-terminal kinase. *J Immunol* 1996; 157:1514-22.
28. Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, Caron MG, Lefkowitz RJ. Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science* 1999; 283:655-61.
29. Hausdorff WP, Lohse MJ, Bouvier M, Liggett SB, Caron MG, Lefkowitz RJ. Two kinases mediate agonist-dependent phosphorylation and desensitization of the beta 2-adrenergic receptor. *Symp Soc Exp Biol* 1990; 44:225-40.
30. Penn RB, Reynold A, Panettieri JR, Benovic JL. Mechanisms of acute desensitization of the β_2AR-adenylyl cyclase pathway in human airway smooth muscle. *Am J Resp Cell Mol Biol* 1998; 19:338- 348.
31. Pitcher JA, Inglese J, Higgins JB, Arriza JL, Casey PJ, Kim C, Benovic JL, Kwatra MM, Caron MG, Lefkowitz RJ. Role of beta gamma subunits of G proteins in targeting the beta-adrenergic receptor kinase to membrane-bound receptors. *Science* 1992; 257:1264-7.
32. Inglese J, Freedman NJ, Koch WJ, Lefkowitz RJ. Structure and mechanism of the G protein-coupled receptor kinases. *J Biol Chem* 1993; 268:23735-8.
33. Meurs H, Kauffinan HF, Koëter GH, Timmermans A, de Vries K. Regulation of the beta-receptor-adenylate cyclase system in lymphocytes of allergic patients with asthma: possible role for protein kinase C in allergen-induced nonspecific refractoriness of adenylate cyclase. *J Allergy Clin Immunol* 1987; 80:326-39.
34. Larsson K, Hjemdahl P, Theodorsson E. Acute bronchoconstriction is not a stimulus for sympatho-adrenal activation in asthmatic or healthy subjects. *Eur Respir J* 1990; 3:273-281.
35. Borger P, Jonker GJ, Vellenga E, Postma DS, De Monchy JGR, Kauffman HF. Allergen challenge primes for IL-5 mRNA production and abrogates beta-adrenergic function in peripheral blood T lymphocytes from asthmatics. *Clin Exp Allergy* 1999; 29:933-40.
36. Aragay AM, Mellado M, Frade JM, Martin AM, Jimenez-Sainz MC, Martinez AC, Mayor F. Monocyte chemoattractant protein-1-induced CCR2B receptor desensitization mediated by the G protein-coupled receptor kinase 2. *Proc Natl Acad Sci USA* 1998; 95:2985-90.
37. Kompa AR, Gu XH, Evans BA, Summers RJ. Desensitization of cardiac beta-adrenoceptor signaling with heart failure produced by myocardial infarction in the rat. Evidence for the role of Gi but not Gs or phosphorylating proteins. *J Mol Cell Cardiol* 1999; 31:1185-201.
38. Tepe NM, Liggett SB. Functional receptor coupling to Gi is a mechanism of agonist-promoted desensitization of the beta2-adrenergic receptor. *J Recept Signal Transduct Res* 2000; 20:75-85.

Chapter 8

Summary, conclusions, discussion and directions for future research

SUMMARY

Bronchial hyperresponsiveness (BHR) and airway inflammation are both characteristic features of asthma. Although methacholine and histamine have become golden standards to assess BHR, an additional level of complexity has been uncovered by using a wide range of indirect stimuli, including Adenosine 5'-Monophosphate (AMP), that elicit bronchoconstriction through the release of spasmogens. Once inhaled, AMP is rapidly converted to adenosine by the ubiquitous enzyme 5'-nucleotidase. A major action of AMP appears to involve the release of histamine and other mediators from immunologically primed mast cells, since AMP-induced bronchoconstriction is associated with a rise of histamine in plasma and bronchoalveolar lavage fluid.[1] Moreover, responsiveness to AMP is inhibited for more than 80% by pretreatment with antihistamines in asthma.[2,3] In **chapter 2**, we have extensively reviewed the role of a provocation test with AMP in asthma and COPD.

The association between airway inflammation and direct bronchial hyperresponsiveness has been the subject of much controversy with almost as many negative as positive reports in the literature.[4-7] Interestingly, it has been suggested that the severity of PC_{20} AMP is more closely associated with airway inflammation than the severity of PC_{20} methacholine, since it has been shown that the PC_{20} AMP improves after a stay of one month in a hypoallergenic environment at high altitude (Switzerland, Davos), whereas PC_{20} methacholine remains stable. However, thus far, the association between the severity of PC_{20} AMP and the extent of airway inflammation has not been directly investigated in the sense of actually collecting data on inflammation in sputum or airway wall biopsies. In **chapter 3**, we have investigated whether the direct association between bronchial hyperresponsiveness and airway inflammation is different for AMP than for methacholine. To this end, we tapered down inhaled corticosteroids in a large group of 120 asthmatic subjects. Thereafter, we assessed the relationship between PC_{20} methacholine as well as PC_{20} AMP with FEV_1 %predicted and inflammatory markers in sputum, blood and exhaled air. When applying multiple regression analysis, we found a dichotomy in the factors explaining the level of PC_{20} methacholine and PC_{20} AMP. The FEV_1 was the most important explanatory variable for the variation in PC_{20} methacholine (explained variance = 18%) with the number of peripheral blood monocytes being a weak additional predictor (total explained variance = 23%). In contrast, the level of PC_{20} AMP was predominantly predicted by the percentage of sputum eosinophils (explained variance = 25%), while FEV_1 was only an additional independent predictor. On the basis of this analysis, we concluded that the PC_{20} AMP better reflects airway inflammation in asthma than the PC_{20} methacholine.

Furthermore, it has been suggested that the PC_{20} AMP is also more sensitive to changes in airway inflammation, since it improves to a greater extent after therapy with corticosteroids than PC_{20} methacholine.[8,9] However, thus far it is unknown whether this greater improvement in PC_{20} AMP is related to a better association of AMP with reduction in airway inflammation than methacholine or more a reflection of different depositions of methacholine and AMP or, alternatively, the fact that more dose steps are being used when provoking with AMP than with methacholine. In **chapter 4**, we have investigated the association between the corticosteroid-induced improvement in PC_{20} AMP and PC_{20}

methacholine on the one hand and the concomitant reduction in airway inflammation on the other hand. The same dataset was used as in chapter 3. Briefly, inhaled corticosteroids were tapered down in 120 patients with mild to moderately severe asthma.[10] Corticosteroids were subsequently started and the relationship assessed between the corticosteroid-induced improvement in both PC_{20} methacholine and PC_{20} AMP and the concomitant increase in the level of FEV_1 %pred and reduction in airway inflammation. The latter was assessed by exhaled air and the number of inflammatory cells and eosinophil cationic protein (ECP) obtained by sputum induction. We found that improvement in PC_{20} AMP was solely related to reduction in airway inflammation (i.e. change in the number of sputum eosinophils, lymphocytes, epithelial cells, and concentration of NO in exhaled air). In contrast, improvement in PC_{20} methacholine was related to both reduction in airway inflammation (i.e. change in the number of sputum eosinophils and lymphocytes) and increase in FEV_1 %pred. Moreover, the total explained variance of the improvement in bronchial hyperresponsiveness was higher for AMP than methacholine (36% versus 22%).We concluded that the PC_{20} AMP is more sensitive to changes in airway inflammation than the PC_{20} methacholine. This again suggests that the PC_{20} AMP is a more powerful tool to monitor active inflammation in the airway wall.

Once inhaled AMP is rapidly converted to adenosine by the ubiquitous enzyme 5'-nucleotidase. It has been suggested in several studies that adenosine, which can also be generated in asthmatic airways itself during the process of energy generation, would contribute to asthma pathogenesis. Thus far, four different adenosine receptors have been described, namely adenosine A_1, A_{2A}, A_{2B}, and A_3 receptors. There is suggestive evidence that AMP-induced bronchoconstriction is mediated by activation of the A_{2B} receptor. In recent years, there has been a tendency to attach less importance to the role of the mast cell as an effector cell in asthma. However, new findings have led to a resurgence of interest in the mast cell and support a reevaluation of its role. In particular, the discovery that mast cells are a source of Th2 type cytokines suggests that mast cell activation can contribute to orchestration of the asthmatic inflammatory response. Adenosine can also act on A_1, A_{2A}, and A_3 receptors, which have been identified on neutrophils, eosinophils, and macrophages. Their activation causes various pro- and anti-inflammatory actions. Surprisingly little is known so far about the exact distribution and function of the various types of adenosine receptors in human airways and hence it is difficult to predict the exact consequences of their activation. Taken together, one could speculate that inhalation of AMP, and by implication adenosine, will initiate an inflammatory response in the airways and this has been investigated in **chapter 5**. We have included 21 asthmatic subjects. Each subject performed three sputum inductions and blood collections on different days at least seven days apart: one without previous provocation, one hour after PC_{20} methacholine, and one hour after PC_{20} AMP. The study was designed in a crossover fashion such that each subject served as his or her own control. To exclude the possibility that our observations could be explained by a non-specific response of the airways to a vigorous bronchoconstriction, we used methacholine as a control challenge. We found that a provocation test with AMP, but not with methacholine, induces an increase in the percentage of sputum eosinophils in patients with asthma. These findings may suggest that adenosine can play a role in chronic inflammatory conditions in asthma either via the release of inflammatory mediators from mast cells or, alternatively, via activation of adenosine A_1, A_{2A}, or A_3 receptors. Since our findings indicate that inhalation of AMP induces an influx of eosinophils into the airways, it could be speculated that a PC_{20} AMP causes a late asthmatic response similar to that seen

after allergen challenge. However, this is unlikely, since previous findings of Phillips and colleagues showed no increase in bronchial hyperresponsiveness to methacholine or a decline in FEV_1 up to 24 hours after AMP challenge.[11]

Interestingly, it has been shown in an animal models that treatment with an A_{2A} receptor agonist has a protective effect against the late asthmatic response after allergen challenge. In **chapter 6**, we investigated whether inhalation of an A_{2A} receptor agonist, which has been developed for human use, will have a similar inhibitory effect on the late asthmatic response (LAR) after allergen in human asthmatic subjects. To investigate this, we performed a double blind, randomized study in 14 atopic asthmatic subjects. We assessed the effects of one week inhaled treatment with either twice daily placebo, an A_{2A} agonist 25 μg, or fluticasone propionate 250μg on the late allergen induced asthmatic response, cell differentiation and inflammatory mediators in induced sputum, and on exhaled nitric oxide. We found that treatment with a dose of 25 μg b.i.d. of this A_{2A}-receptor agonist did not protect against the late allergen-induced asthmatic response nor the associated increase in airway inflammation in humans, whereas fluticasone propionate did so as a positive control.

Activation of β_2-receptors inhibits the release of Th2 type cytokines such as IL-5 from peripheral blood lymphocytes. Further, it has been shown that β_2-receptors desensitize after repeated stimulation, thereby explaining why regular treatment with short-acting β_2-receptor agonists as single treatment does not have any beneficial effect in asthma. In addition, it has been suggested that β_2-receptors also become desensitized after exposure to inflammatory mediators, probably via activation of protein kinase C. Taken together, it may be interesting to speculate that an impaired negative feedback control by the β_2 adrenergic system could be involved in the enhanced cellular activity after allergen challenge. In **chapter 7**, we have investigated whether the β_2-adrenergic regulation of peripheral blood lymphocytes in asthmatic subjects is changed from before to 6 hours after allergen challenge. Cytokine mRNA expression patterns were studied in α-CD3/α-CD28 stimulated peripheral lymphocytes in presence and absence of the β_2-agonist fenoterol, using semi-quantitative RT-PCR (LightCycler). In addition, we studied the effect of fenoterol on T cell chemotaxis and signaling pathways. We found a complete loss of β_2-adrenergic control over the Th2 cytokines IL-4, IL-5 and IL-13, but not the Th1 cytokine IFN-γ after challenge. Our findings indicate that the negative feedback control by the β_2-adrenergic system is impaired after allergen challenge and this could contribute to the enhanced cellular activity after allergen challenge

CONCLUSIONS

The main conclusions derived from the studies presented in this thesis are:

- The PC_{20} methacholine is barely associated with airway inflammation in asthma.
- The PC_{20} AMP is more closely associated with airway inflammation (as reflected by the percentage of sputum eosinophils) in asthma than the PC_{20} methacholine.
- Corticosteroid-induced changes in PC_{20} AMP are more closely associated with concomitant changes in airway inflammation (as reflected by changes in the percentages of inflammatory cells in induced sputum) than corticosteroid-induced changes in PC_{20} methacholine.
- The cell differential count in induced sputum does not change one hour after a PC_{20} methacholine.
- The percentage of sputum eosinophils increases one hour after a PC_{20} AMP.
- One week treatment with the inhaled A_{2A} agonist (GW328267X) 25 μg twice daily does not protect against the late fall in FEV_1 after allergen challenge (between 3 and 8 hours after the early asthmatic response upon allergen challenge).
- One week treatment with the inhaled A_{2A} agonist (GW328267X) 25 μg twice daily does not protect against the increase in airway inflammation 24 hours after allergen challenge.
- The β_2-adrenergic control over production of the Th2 cytokines IL-4, IL-5 and IL-13, but not the Th1 cytokine IFN-γ in peripheral blood lymphocytes is completely lost 6 hours after an allergen challenge.

DISCUSSION AND DIRECTIONS FOR FUTURE RESEARCH

PC_{20} AMP as a non-invasive tool to monitor airway inflammation in asthma.

We have demonstrated for the first time directly that the PC_{20} AMP is associated with airway inflammation as reflected by the percentage of eosinophils in sputum and blood, whereas this is less explicit with the PC_{20} methacholine.[12] More importantly, we have shown that the steroid-induced improvement of PC_{20} AMP is also more closely related to the concomitant reduction in airway inflammation than the PC_{20} methacholine.[13] Together, these findings suggest that the PC_{20} AMP can be useful as a non-invasive tool to monitor airway inflammation in asthma.

This may be important, since it has been demonstrated by Sont and colleagues that a treatment algorithm incorporating PC_{20} methacholine, as a surrogate marker of airway inflammation, can improve lung function (i.e. FEV_1) and also reduce basement membrane thickening, the end result of inflammation.[14] Thus, directing treatment simply at improvement of symptoms and lung function does not lead to optimal asthma control. However, patients in the latter study who were treated based on the level of PC_{20} methacholine used higher doses of inhaled corticosteroids, which hampers the interpretation of the results. Furthermore, PC_{20} methacholine was used as a surrogate marker of airway inflammation, which in the light of current data of this thesis appears not to be fully valid.

In a recent study of Green and colleagues, a treatment strategy directed to normalize the percentage of sputum eosinophils was compared with one guided by symptoms and lung function (BTS guidelines).[15] Patients who received adjustment of anti-inflammatory treatment based on the percentage of sputum eosinophils showed a remarkable improvement in asthma control with a lower level of bronchial hyperresponsiveness and a reduction in the number of asthma exacerbations. In addition, the doses of inhaled corticosteroids did not differ between the two groups. Thus, this is the first study undisputedly showing that monitoring of airway inflammation may be important to improve the management and outcome of asthma. Despite this positive notion, sputum induction is unlikely to have a place in asthma management in the foreseeable future given the drawbacks that limit its use in daily clinical practice. Dedicated, highly qualified laboratory technicians are needed and the technique is time consuming, preventing rapid help in decision making to change treatment. The sputum induction procedure takes at maximum one hour and processing up to the necessary provision of percentage eosinophils approximately three hours.

Thus, it is now clear that further development and validation of other noninvasive markers of airway inflammation becomes an important research goal. It could be speculated that treatment based on the severity of PC_{20} AMP leads to a better control of (eosinophilic) inflammation in asthma when compared to treatment based on symptoms and lung function only. This has to be sorted out in future studies.

Role of mast cells in asthma

We have shown in chapter 5 of this thesis that a provocation test with AMP, but not with methacholine, induces an inflammatory response in the airways of asthmatic patients as reflected by an increase in the percentage of sputum eosinophils. There is suggestive evidence that AMP-induced bronchoconstriction is mediated by the release of inflammatory mediators from mast cells. In recent years, there has been a tendency to attach less importance to the role of the mast cell as an effector cell in asthma. However, new findings have led to a resurgence of interest in the mast cell and support a reevaluation of its role. In particular, the discovery that mast cells are a source of Th2 type cytokines has led to the suggestion that mast cell activation can contribute to the orchestration of the asthmatic inflammatory response.[16-18] However, the exact role of mast cells in the orchestration of airway inflammation remains to be elucidated.

The role of mast cells in asthma could be further investigated in 'in vitro' studies. Thus far, a major limitation in performing 'in vitro' studies in mast cells has been the difficulty to obtain a culture of human mast cells from adult subjects with or without asthma.[19] For this reason, previous studies have used mast cells derived from cord blood or from a patient with mast cell leukemia.[20,21] Whether mast cells cultured from cord blood or derived from a patient with mast leukemia resemble normal human mast cells is questionable, since it has been shown that they do not express FcεR1 receptors.[22,23] Recently, it has been shown to be possible to culture human mast cells from peripheral blood. Eight weeks culture of mast cell progenitors in peripheral blood with SCF, IL-3, and IL-6 resulted in > 95% pure mast cell colonies.[22,24] Obviously, this creates new opportunities to investigate the role of mast cells in asthma. For example, it now becomes possible to investigate exactly which cytokines are released upon activation of mast cells. Furthermore, the time course of the different cytokines and mediators released could be elucidated. Finally, the immunologic pathways by which mast cells interact with other inflammatory cells could be studied. Together, this would markedly enhance our knowledge about the role of mast cells in the regulation of the asthmatic inflammatory response.

Provocation with AMP may become another tool for further exploration of the role of mast cells in asthma, since there is now convincing evidence that the airway response to this nucleotide is an index of mast cell number and/or activity. In this context, the findings of O'Connor and colleagues are of special interest.[25] They found that inhaled β_2-receptor agonists, when given as single dose, provide greater bronchoprotection against AMP-induced bronchoconstriction than against methacholine-induced bronchoconstriction and this differential bronchoprotective effect has been interpreted as mast cell stabilization.[25] In agreement with this, it has been demonstrated that β_2-receptor agonists inhibit the release of histamine from chopped human lung and dispersed human lung mast cells.[26,27] Further, inhaled albuterol has been shown to inhibit the early increase in plasma histamine induced by allergen exposure in asthmatic patients, although it does not inhibit the late allergen-induced decline in FEV_1.[28] The protection provided by β_2-agonists against mast cell mediator release might be important to prevent acute exacerbations in asthma, which are partly mediated by mast cell activation.[29] This action may contribute to the reduction in severe and mild exacerbations seen when formoterol is added to maintenance treatment with inhaled corticosteroids.[30] Unfortunately, no studies are available about the additive effect of salmeterol in asthmatic patients already using inhaled corticosteroids. Thus far,

studies investigating the additive effect of salmeterol have compared this with the additive effect of a higher dose of inhaled corticosteroids.[31] An argument against the explanation as to the beneficial effect of combination therapy with formoterol and budesonide could be that subsensitivity to the bronchoprotective effect against AMP develops when asthma patients use formoterol regularly for one month.[32] These findings suggest that β_2-receptors present on mast cells desensitize rapidly after daily treatment with formoterol. Although corticosteroids prevent the loss of function of β_2-agonists with chronic use, this effect also occurs despite concomitant inhaled corticosteroid therapy.[33-35] It has been reported that the extent of desensitization is highly variable between subjects and these interindividual differences are linked to genetic polymorphisms in the β_2-receptor.[36] Thus, it could be speculated that a subgroup of asthma patients is resistant to mast cell β_2-receptor desensitization. In this subgroup of asthma patients, adding formoterol to maintenance therapy with inhaled corticosteroids may well have an additional anti-inflammatory effect. Taken together, it now becomes interesting to investigate whether polymorphisms of the β_2-receptor could identify a subgroup of asthma patients that is resistant to the development of subsensitivity to the differential protective effect of β_2-receptor agonists.

Adenosine as a mediator of inflammation in asthma

Adenosine is a naturally occurring purine nucleoside with a ubiquitous presence in human tissue. AMP is formed by the catabolism of high-energy adenosine phosphates such as adenosine diphosphate (ADP) or adenosine tri-phosphate (ATP) during the process of energy generation. When sufficient oxygen and energy is available, AMP is reconverted to high-energy phosphates forming a component of the energy cycle.[37] However, in case of excessive cell stimulation or hypoxia, AMP can not be reconverted and is transported to the exterior of the cell where it is metabolized to adenosine by the enzyme 5'-nucleotidase. The discovery that adenosine levels are elevated in the bronchoalveolar lavage fluid and in exhaled breath of asthmatics and increase further after allergen challenge raises the possibility that adenosine generated in asthmatic airway could itself contribute to the pathogenesis of asthma.[38-40] Another interesting finding in this context is that mice which lack the enzyme adenosine deaminase (and are therefore unable to break off endogenously formed adenosine) develop features of asthma including bronchial hyperresponsiveness, enhanced mucus secretion, airway eosinophilia, increased IgE synthesis, and elevated bronchoalveolar levels of IL-5.[41,42] All these asthmatic features could be reversed after administration of adenosine deaminase. Transcript array technology has been used to examine which genes in the lung become activated by adenosine that accumulates in adenosine deaminase deficient mice. This has provided additional insights into mechanisms linking adenosine to asthmatic airway inflammation by revealing marked overexpression of the monocyte chemotactic protein-3 gene in the airways paralleled by enhanced protein secretion. Monocyte chemotactic protein-3 is a chemokine with powerful eosinophil chemotactic properties. In agreement with the above findings, we have demonstrated that inhalation of AMP, and by implication adenosine, results in a rapid (within one hour) increase in sputum eosinophils.

Adenosine may contribute to airway inflammation via activation of four different adenosine receptors, namely adenosine A_1, A_{2A}, A_{2B}, and A_3 receptors. Human lung mast cells have been shown to express A_{2A} and A_{2B} receptors, but not A_1 or A_3 receptors.[43,44] In a recent study in cultured mast cells derived from a patient with mast cell leukemia, only activation

of the A_{2B} receptor resulted in mast cell activation.[45] Thus, there is now suggestive evidence that AMP-induced bronchoconstriction is mediated by activation of the A_{2B} receptor. Once inhaled, adenosine can also act on A_1, A_{2A}, and A_3 receptors, which have been identified on neutrophils, eosinophils, macrophages, and endothelial cells. Their activation causes various pro- and anti-inflammatory actions: activation of adenosine A_1 receptors promotes chemotaxis of neutrophils and increases adherence of neutrophils to endothelial cells.[46-48] By contrast, activation of adenosine A_{2A} receptors reduces chemotaxis, activation and degranulation of neutrophils.[47,49,50] In addition, activation of A_{2A} receptors (in contrast to activation of A_{2B} receptors) suppresses the release of tryptase from mast cells.[51] A_3 receptors mediate inhibition of eosinophil chemotaxis when activated.[43] Other functions of A_3 receptors involve inhibition of neutrophil degranulation induced by LPS or TNF-α. Surprisingly little is known so far about the exact distribution and function of the various types of adenosine receptors in human airways and hence it is difficult to predict the exact consequences of their activation. Thus, it is important to further elucidate the role of adenosine and adenosine receptors in asthma. This could be done in several ways. Firstly, it may be interesting to sequence adenosine A_1, A_{2A}, A_{2B}, and A_3 receptors and the enzyme adenosine deaminase. Then, it becomes possible to investigate whether polymorphisms are associated with the level of AMP responsiveness or more severe asthma (i.e. a more severe bronchial hyperresponsiveness to methacholine, a lower level of lung function, higher numbers of inflammatory cells in bronchial biopsies, bronchoalveolar lavage fluid, sputum, or blood, or the number of asthma exacerbations). One can also investigate whether polymorphisms in the adenosine receptor are functional and which consequences it has for receptor and postreceptor mechanisms. Secondly, it may be interesting to investigate whether adenosine receptors on inflammatory cells become upregulated by inflammatory mediators. This could be performed either 'in vitro' in cell culture systems, or 'in vivo' by assessing the number of adenosine A_1, A_{2A}, A_{2B}, and A_3 receptors on inflammatory cells derived from induced sputum before and after allergen challenge. Upregulation of adenosine receptors by inflammatory mediators could help to explain our findings of an association between the severity of PC_{20} AMP and the extent of airway inflammation. Thirdly, it may be of interest to investigate adenosine receptors present on human mast cells which are cultured from peripheral blood. Thus, it could be elucidated whether there are differences in mediators released from mast cells after activation of adenosine receptors on the one hand and FcεR1 receptors on the other hand. Further, it could be investigated whether the adenosine-induced release of mediators from mast cells is inhibited by treatment with (combinations of) an inhaled corticosteroid, salmeterol, formoterol, albuterol, and nedocromil. Fourthly, it may be possible to use animal models to further elucidate the role of adenosine as a mediator of inflammation in asthma. For example, it would be interesting to investigate in sensitized mice whether repeated AMP provocations will result in an increase in airway inflammation as reflected by the number of eosinophils in lung tissue and to assess whether this increase in inflammation is inhibited by treatment with corticosteroids. Fifthly, it would be interesting to develop knock-in and knock-out mice, in which the adenosine A_1, A_{2A}, A_{2B}, and A_3 can be switched on and off. This will enable us to enhance our understanding of the exact role of the different adenosine receptors in asthma. Finally, it becomes important to develop drugs which are designed to specifically inhibit (A_1, A_{2B}) or activate (A_3, A_{2A}) adenosine receptors and to investigate whether these drugs could serve as an appropriate asthma treatment, since it has been shown that adenosine A_1 inhibitors and adenosine A_{2A} agonists provide a protective effect on the late asthmatic response after allergen challenge in an animal model.[52] In this context

our study with a newly developed A_{2A} adenosine receptor agonist was a step forward, although we could not demonstrate a protective effect against the late asthmatic response after allergen challenge.

Thus, studies in this thesis may lead to a better understanding of the pathogenesis of asthma, and specifically the role of mast cells. In addition, studies in this thesis may lead to options for better asthma management by using AMP to titrate asthma management, although this has to be investigated in future studies.

REFERENCES

1. Polosa R, Ng WH, Crimi N, Vancheri C, Holgate ST, Church MK, Mistretta A. Release of mast-cell-derived mediators after endobronchial adenosine challenge in asthma. *Am J Respir Crit Care Med* 1995; 151:624-9.
2. Rutgers SR, Koëter GH, van der Mark TW, Postma DS. Protective effect of oral terfenadine and not inhaled ipratropium on adenosine 5'-monophosphate-induced bronchoconstriction in patients with COPD. *Clin Exp Allergy* 1999; 29:1287-92.
3. Polosa R, Ciamarra I, Mangano G, Prosperini G, Pistorio MP, Vancheri C, Crimi N. Bronchial hyperresponsiveness and airway inflammation markers in nonasthmatics with allergic rhinitis. *Eur Respir J* 2000; 15:30-5.
4. Aalbers R, De Monchy JG, Kauffman HF, Smith M, Hoekstra Y, Vrugt B, Timens W. Dynamics of eosinophil infiltration in the bronchial mucosa before and after the late asthmatic reaction. *Eur Respir J* 1993; 6:840-7.
5. Alvarez MJ, Olaguibel JM, Garcia BE, Rodriquez A, Tabar AI, Urbiola E. Airway inflammation in asthma and perennial allergic rhinitis. Relationship with nonspecific bronchial responsiveness and maximal airway narrowing. *Allergy* 2000; 55:355-62.
6. Crimi E, Spanevello A, Neri M, Ind PW, Rossi GA, Brusasco V. Dissociation between airway inflammation and airway hyperresponsiveness in allergic asthma. *Am J Respir Crit Care Med* 1998; 157:4-9.
7. Bradley BL, Azzawi M, Jacobson M, Assoufi B, Collins JV, Irani AM, Schwartz LB, Durham SR, Jeffery PK, Kay AB. Eosinophils, T-lymphocytes, mast cells, neutrophils, and macrophages in bronchial biopsy specimens from atopic subjects with asthma: comparison with biopsy specimens from atopic subjects without asthma and normal control subjects and relationship to bronchial hyperresponsiveness. *J Allergy Clin Immunol* 1991; 88:661-74.
8. Weersink EJ, Douma RR, Postma DS, Koëter GH. Fluticasone propionate propionate, salmeterol xinafoate, and their combination in the treatment of nocturnal asthma. *Am J Respir Crit Care Med* 1997; 155:1241-6.
9. O'Connor BJ, Ridge SM, Barnes PJ, Fuller RW. Greater effect of inhaled budesonide on adenosine 5'-monophosphate- induced than on sodium-metabisulfite-induced bronchoconstriction in asthma. *Am Rev Respir Dis* 1992; 146:560-4.
10. Meijer RJ, Kerstjens HAM, Arends LR, Kauffman HF, Koëter GH, Postma DS. Effects of inhaled fluticasone propionate and oral prednisolone on clinical and inflammatory parameters in patients with asthma. *Thorax* 1999; 54:894-9.
11. Phillips GD, Holgate ST. Absence of a late-phase response or increase in histamine responsiveness after bronchial provocation with adenosine 5'- monophosphate in atopic and non-atopic asthma. *Clin Sci* 1988; 75:429-36.
12. Van Den Berge M, Meijer RJ, Kerstjens HAM, De Reus DM, Koëter GH, Kauffman HF, Postma DS. PC_{20} adenosine 5'-monophosphate is more closely associated with airway inflammation in asthma than PC_{20} methacholine. *Am J Respir Crit Care Med* 2001; 163:1546-50.
13. Van Den Berge M, Kerstjens HAM, Meijer RJ, De Reus DM, Koëter GH, Kauffman HF, Postma DS. Corticosteroid-induced Improvement in the PC_{20} of Adenosine Monophosphate Is More Closely Associated with Reduction in Airway Inflammation than Improvement in the PC_{20} of Methacholine. *Am J Respir Crit Care Med* 2001; 164:1127-32.
14. Sont JK, Willems LN, Bel EH, van Krieken JH, Vandenbroucke JP, Sterk PJ. Clinical control and histopathologic outcome of asthma when using airway hyperresponsiveness as an additional guide to long-term treatment. The AMPUL Study Group. *Am J Respir Crit Care Med* 1999; 159:1043-51.
15. Green RH, Brightling CE, McKenna S, Hargadon B, Parker D, Bradding P, Wardlaw AJ, Pavord ID. Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. *Lancet* 2002; 360:1715-21.
16. Bradding P, Holgate ST. The mast cell as a source of cytokines in asthma. *Ann N Y Acad Sci* 1996; 796:272-81.
17. Bradding P, Roberts JA, Britten KM, Montefort S, Djukanovic R, Mueller R, Heusser CH, Howarth PH, Holgate ST. Interleukin-4, -5, and -6 and tumor necrosis factor-alpha in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines. *Am J Respir Cell Mol Biol* 1994; 10:471-80.
18. Bentley AM, Meng Q, Robinson DS, Hamid Q, Kay AB, Durham SR. Increases in activated T lymphocytes, eosinophils, and cytokine mRNA expression for interleukin-5 and granulocyte/macrophage colony-stimulating factor in bronchial biopsies after allergen inhalation challenge in atopic asthmatics. *Am J Respir Cell Mol Biol* 1993; 8:35-42.
19. Okayama Y. Human cultured mast cells. *Clin Exp Allergy* 2000; 30:1053-5.

20. Boyce JA, Mellor EA, Perkins B, Lim YC, Luscinskas FW. Human mast cell progenitors use alpha4-integrin, VCAM-1, and PSGL-1 E- selectin for adhesive interactions with human vascular endothelium under flow conditions. *Blood* 2002; 99:2890-6.
21. Hosoda M, Yamaya M, Suzuki T, Yamada N, Kamanaka M, Sekizawa K, Butterfield JH, Watanabe T, Nishimura H, Sasaki H. Effects of rhinovirus infection on histamine and cytokine production by cell lines from human mast cells and basophils. *J Immunol* 2002; 169:1482-91.
22. Iida M, Matsumoto K, Tomita H, Nakajima T, Akasawa A, Ohtani NY, Yoshida NL, Matsui K, Nakada A, Sugita Y, Shimizu Y, Wakahara S, Nakao T, Fujii Y, Ra C, Saito H. Selective down-regulation of high-affinity IgE receptor (FcepsilonRI) alpha-chain messenger RNA among transcriptome in cord blood-derived versus adult peripheral blood-derived cultured human mast cells. *Blood* 2001; 97:1016-22.
23. Xia HZ, Kepley CL, Sakai K, Chelliah J, Irani AM, Schwartz LB. Quantitation of tryptase, chymase, Fc epsilon RI alpha, and Fc epsilon RI gamma mRNAs in human mast cells and basophils by competitive reverse transcription-polymerase chain reaction. *J Immunol* 1995; 154:5472-80.
24. Welker P, Grabbe J, Zuberbier T, Guhl S, Henz BM. Mast cell and myeloid marker expression during early in vitro mast cell differentiation from human peripheral blood mononuclear cells. *J Invest Dermatol* 2000; 114:44-50.
25. O'Connor BJ, Fuller RW, Barnes PJ. Nonbronchodilator effects of inhaled beta 2 agonists. Greater protection against adenosine monophosphate- than methacholine-induced bronchoconstriction in asthma. *Am J Respir Crit Care Med* 1994; 150:381-7.
26. Peters SP, Schulman ES, Schleimer RP, Macglashan DW, Jr., Newball HH, Lichtenstein LM. Dispersed human lung mast cells. Pharmacologic aspects and comparison with human lung tissue fragments. *Am Rev Respir Dis* 1982; 126:1034-9.
27. Church MK, Hiroi J. Inhibition of IgE-dependent histamine release from human dispersed lung mast cells by anti-allergic drugs and salbutamol. *Br J Pharmacol* 1987; 90:421-9.
28. Howarth PH, Durham SR, Lee TH, Kay AB, Church MK, Holgate ST. Influence of albuterol, cromolyn sodium and ipratropium bromide on the airway and circulating mediator responses to allergen bronchial provocation in asthma. *Am Rev Respir Dis* 1985; 132:986-92.
29. Barnes PJ. Scientific rationale for inhaled combination therapy with long-acting beta2-agonists and corticosteroids. *Eur Respir J* 2002; 19:182-91.
30. Pauwels RA, Lofdahl CG, Postma DS, Tattersfield AE, O'Byrne P, Barnes PJ, Ullman A. Effect of inhaled formoterol and budesonide on exacerbations of asthma. Formoterol and Corticosteroids Establishing Therapy (FACET) International Study Group. *N Engl J Med* 1997; 337:1405-11.
31. Greening AP, Ind PW, Northfield M, Shaw G. Added salmeterol versus higher-dose corticosteroid in asthma patients with symptoms on existing inhaled corticosteroid. Allen & Hanburys Limited UK Study Group. *Lancet* 1994; 344:219-24.
32. Rowe BH, Keller JL, Oxman AD. Effectiveness of steroid therapy in acute exacerbations of asthma: a meta-analysis. *Am J Emerg Med* 1992; 10:301-10.
33. Aziz I, Tan KS, Hall IP, Devlin MM, Lipworth BJ. Subsensitivity to bronchoprotection against adenosine monophosphate challenge following regular once-daily formoterol. *Eur Respir J* 1998; 12:580-4.
34. O'Connor BJ, Aikman SL, Barnes PJ. Tolerance to the nonbronchodilator effects of inhaled beta 2-agonists in asthma. *N Engl J Med* 1992; 327:1204-8.
35. Barnes PJ. Scientific rationale for inhaled combination therapy with long-acting beta2-agonists and corticosteroids. *Eur Respir J* 2002; 19:182-91.
36. Chong LK, Morice AH, Yeo WW, Schleimer RP, Peachell PT. Functional desensitization of beta agonist responses in human lung mast cells. *Am J Respir Cell Mol Biol* 1995; 13:540-6.
37. Polosa R, Rorke S, Holgate ST. Evolving concepts on the value of adenosine hyperresponsiveness in asthma and chronic obstructive pulmonary disease. *Thorax* 2002; 57:649-54.
38. Driver AG, Kukoly CA, Ali S, Mustafa SJ. Adenosine in bronchoalveolar lavage fluid in asthma. *Am Rev Respir Dis* 1993; 148:91-7.
39. Mann JS, Holgate ST, Renwick AG, Cushley MJ. Airway effects of purine nucleosides and nucleotides and release with bronchial provocation in asthma. *J Appl Physiol* 1986; 61:1667-76.
40. Mann JS, Holgate ST, Renwick AG, Cushley MJ. Airway effects of purine nucleosides and nucleotides and release with bronchial provocation in asthma. *J Appl Physiol* 1986; 61:1667-76.
41. Chunn JL, Young HW, Banerjee SK, Colasurdo GN, Blackburn MR. Adenosine-dependent airway inflammation and hyperresponsiveness in partially adenosine deaminase-deficient mice. *J Immunol* 2001; 167:4676-85.
42. Zhong H, Chunn JL, Volmer JB, Fozard JR, Blackburn MR. Adenosine-mediated mast cell degranulation in adenosine deaminase- deficient mice. *J Pharmacol Exp Ther* 2001; 298:433-40.
43. Walker BA, Jacobson MA, Knight DA, Salvatore CA, Weir T, Zhou D, Bai TR. Adenosine A_3 receptor expression and function in eosinophils. *Am J Respir Cell Mol Biol* 1997; 16:531-7.

44. Marquardt R, Fischer J, Kustner W. Effect of arterial hypoxia and acidosis on fibrillation threshold in the cat heart in vivo. *Z Kardiol* 1976; 65:585-9.
45. Feoktistov I, Biaggioni I. Adenosine A_{2B} receptors evoke interleukin-8 secretion in human mast cells. An enprofylline-sensitive mechanism with implications for asthma. *J Clin Invest* 1995; 96:1979-86.
46. Cronstein BN, Daguma L, Nichols D, Hutchison AJ, Williams M. The adenosine/neutrophil paradox resolved: human neutrophils possess both A_1 and A_2 receptors that promote chemotaxis and inhibit O_2 generation, respectively. *J Clin Invest* 1990; 85:1150-7.
47. Cronstein BN, Levin RI, Philips M, Hirschhorn R, Abramson SB, Weissmann G. Neutrophil adherence to endothelium is enhanced via adenosine A_1 receptors and inhibited via adenosine A_2 receptors. *J Immunol* 1992; 148:2201-6.
48. Felsch A, Stocker K, Borchard U. Phorbol ester-stimulated adherence of neutrophils to endothelial cells is reduced by adenosine A_2 receptor agonists. *J Immunol* 1995; 155:333-8.
49. Wollner A, Wollner S, Smith JB. Acting via A_2 receptors, adenosine inhibits the upregulation of Mac-1 (Cd11b/CD18) expression on FMLP-stimulated neutrophils. *Am J Respir Cell Mol Biol* 1993; 9:179-85.
50. Fredholm BB, Zhang Y, van dP, I. Adenosine A_{2A} receptors mediate the inhibitory effect of adenosine on formyl-Met-Leu-Phe-stimulated respiratory burst in neutrophil leucocytes. *Naunyn Schmiedebergs Arch Pharmacol* 1996; 354:262-7.
51. Suzuki H, Takei M, Nakahata T, Fukamachi H. Inhibitory effect of adenosine on degranulation of human cultured mast cells upon cross-linking of Fc epsilon RI. *Biochem Biophys Res Commun* 1998; 242:697-702.
52. Fozard JR, Ellis KM, Villela Dantas MF, Tigani B, Mazzoni L. Effects of CGS 21680, a selective adenosine A_{2A} receptor agonist, on allergic airways inflammation in the rat. *Eur J Pharmacol* 2002; 438:183-8.

Chapter 9

Nederlandse samenvatting

INLEIDING

Astma is een luchtwegaandoening die wordt gekenmerkt door aanvallen van kortademigheid, piepen op de borst en hoesten. Deze klachten kunnen sterk in intensiteit wisselen en variëren zowel over de dag als van dag tot dag. Voor het vaststellen van astma maakt men gebruik van verschillende longfunctietesten Een belangrijke longfunctietest is de meting van de FEV_1, dit is het volume dat de eerste seconde van de uitademing maximaal kan worden uitgeblazen. Een verlaagde FEV_1 duidt op luchtwegvernauwing (obstructie) tijdens de uitademing. Kenmerkend voor astmapatiënten is dat de FEV_1 gedurende klachtenvrije perioden normaal is maar tijdens perioden mèt klachten verlaagd. Daarnaast zijn veel mensen met astma allergisch voor ingeademde deeltjes van bijvoorbeeld huisstofmijt, huidschilfers van katten of honden, en stuifmeel van bloemen, grassen en bomen, de zogenaamde allergenen.

LUCHTWEGREACTIVITEIT

We spreken van een verhoogde luchtwegreactiviteit indien de luchtwegen zich vernauwen na blootstelling aan een bepaalde hoeveelheid niet-allergische prikkels, die geen reactie geeft bij gezonde mensen. Een verhoogde luchtwegreactiviteit kan bij vrijwel alle astmapatiënten worden aangetoond die geen medicijnen gebruiken. Klinisch uit dit zich in het ontstaan van kortademigheid na expositie aan bijvoorbeeld koude lucht, sigarettenrook, mist, parfumluchtjes en na het verrichten van inspanning. De mate van luchtwegreactiviteit geeft een indruk over de ernst van het astma en wordt gemeten met behulp van een zogenaamde inhalatie provocatietest. Tijdens een dergelijke test inhaleren mensen via een gestandaardiseerde vernevelaar en gedurende een vaste tijd (bijvoorbeeld twee minuten) oplopende concentraties van prikkelende stoffen zoals histamine of methacholine. Na iedere concentratiestap wordt het effect van histamine of methacholine op de longfunctie (FEV_1) bepaald. De concentratie histamine of methacholine die een daling van 20% ten opzichte van de uitgangswaarde van de FEV_1 veroorzaakt wordt de PC_{20} waarde genoemd. Indien 20% daling is bereikt of de maximale concentratie is gegeven, wordt de test gestopt en krijgen patiënten een luchtwegverwijder toegediend om de longfunctie te herstellen.

LUCHTWEGONTSTEKING BIJ ASTMA

Astma-aanvallen worden uitgelokt door blootstelling aan allergenen en/of virussen. Door inhalatie van allergenen ontstaat een ontstekingsreactie in de luchtwegen, de allergische inflammatie. Tijdens dit ontstekingsproces bevinden zich veel ontstekingscellen in de luchtwegwand zoals mestcellen, eosinofiele granulocyten, neutrofiele granulocyten, lymfocyten, en macrofagen. Tevens zijn de wanden van de bloedvaatjes verhoogd doorlaatbaar, waardoor plasma (niet cellulaire component van het bloed) in het omringende weefsel van de luchtwegwand lekt. Dit alles zorgt voor een verdikte luchtwegwand. Daarnaast trekken de spiertjes rondom de luchtwegwand zich samen waardoor de doorgankelijkheid van de luchtwegen afneemt. Voor bestudering van het ontstekingsproces in de luchtwegen wordt vaak gebruik gemaakt van longslijm (sputum). In praktijk blijkt dat veel patiënten het moeilijk vinden sputum spontaan op te hoesten. Door patiënten zoute

nevel te laten inhaleren wordt sputum ophoesten makkelijker, maar dit proces is tijdrovend en analyse van het opgewekt sputum kostbaar.

Vroege en late allergische reactie

De reactie van een astmapatiënt op allergenen kan in 2 stadia worden onderverdeeld, namelijk de vroege en de late reactie. Wanneer een astmapatiënt in contact is geweest met allergische prikkels, kan dit na tien tot twintig minuten tot luchtwegvernauwing leiden (vroege reactie). Deze verdwijnt na ongeveer een uur doorgaans. Een vroege reactie berust voor een groot deel op contractie van het spierweefsel rondom de luchtwegen. Bij ongeveer 70% van de patiënten treedt er na vier tot zes uur opnieuw luchtwegvernauwing op (late reactie) en deze kan in veel gevallen tot meer dan 24 uur aanhouden. De late reactie is het gevolg van ontstekingsprocessen en kan voor een belangrijk deel voorkomen worden met ontstekingsremmende inhalatiemedicijnen.

BEHANDELING VAN ASTMA

De behandeling van astma is gebaseerd op 2 principes, namelijk het bestrijden van symptomen met luchtwegverwijders en het behandelen van het onderliggend ontstekingsproces met ontstekingremmers.

Luchtwegverwijders

Tijdens een astma-aanval trekken de spieren rondom de luchtwegen samen, waardoor luchtwegvernauwing ontstaat. Luchtwegverwijders ($ß_2$-agonisten) stimuleren de $ß_2$-receptor van de gladde spiercellen en zorgen daarmee voor relaxatie (ontspanning) van het gladde spierweefsel. Hierdoor verbeteren de symptomen van een patiënt vrijwel direct; het onderliggend ontstekingsproces wordt er echter bij mensen waarschijnlijk niet door geremd.

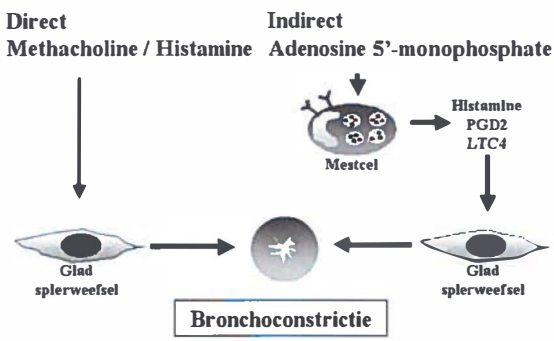
Ontstekingsremmers

Het ontstekingsproces wordt bestreden met ontstekingsremmende inhalatiemedicijnen. Het bekendste voorbeeld is inhalatiecorticosteroïden, welke worden geleverd onder de merknamen Flixotide, Pulmicort, Becotide, en Qvar. Na gebruik van ontstekingsremmers neemt het aantal ontstekingscellen in de luchtwegwand af, herstellen de bloedvaatjes en vermindert de luchtwegvernauwing. Ook verminderen de luchtwegreactiviteit en de klachten. Voor het bepalen van de juiste dosering ontstekingsremmers wordt in praktijk met name gekeken naar de ernst van de klachten, eventueel in combinatie met de longfunctie. Er zijn echter studies die aantonen dat klachten onvoldoende betrouwbaar zijn voor het inschatten van de ernst van het astma. Klachten geven vooral weer in hoeverre de aandoening hinderlijk is voor de patiënt en niet de werkelijke ernst van de aandoening. Het is aangetoond dat sommige astmapatiënten geen klachten rapporteren, ondanks de aanwezigheid van forse ontstekingsactiviteit in de luchtwegen. Ook in afwezigheid van klachten is het echter belangrijk het astmatische ontstekingsproces te behandelen, onder andere omdat het de longen van astmapatiënten gevoeliger maakt voor virusinfecties. Verder zijn er aanwijzingen dat de longfunctie van ongeveer 20% van de astmapatiënten versneld afneemt, in vergelijking met een gezonde groep mensen. Dit wordt voor een

belangrijk deel toegeschreven aan het aanwezige ontstekingsproces en de daardoor optredende structurele veranderingen in de luchtwegwand. In de afgelopen jaren is daarom veel onderzoek verricht naar een methode voor het meten van luchtwegontsteking. Op die manier zou het mogelijk moeten zijn de dosering inhalatiesteroïden vast te stellen op basis van de ontstekingsactiviteit van de luchtwegen; dit zou een belangrijke stap vooruit zijn bij de behandeling van astma.

EEN PROVOCATIETEST MET AMP ALS MARKER VOOR LUCHTWEGONTSTEKING

Een mogelijke manier voor het meten van luchtwegontsteking, is een provocatietest met Adenosine 5'-Monofosfaat (AMP). De werking van AMP in de luchtwegen is anders dan die van histamine of methacholine. Histamine en methacholine hebben een direct effect op het gladde spierweefsel van de luchtwegwand en zorgen zo voor een vernauwing van de luchtwegen (bronchoconstrictie). AMP heeft geen direct effect op het gladde spierweefsel, maar werkt indirect via het vrijmaken van ontstekingseiwitten (mediatoren) uit ontstekingscellen (namelijk mestcellen). Deze mediatoren zorgen vervolgens voor luchtwegvernauwing (zie figuur). In de kliniek en in het wetenschappelijk onderzoek naar astma bestaat een groeiende interesse voor een provocatietest met AMP, omdat recente studies aannemelijk maken dat deze test de mate van luchtwegontsteking in astma beter weerspiegelt dan een provocatietest met methacholine. Zo is het bijvoorbeeld aangetoond dat de verbetering van astma na een verblijf van een maand in een allergeenarme omgeving op grote hoogte (in Davos, Zwitserland) wel aangetoond kan worden met de PC_{20} AMP, maar niet met de PC_{20} methacholine. In **hoofdstuk 2** wordt een uitgebreid literatuuroverzicht gepresenteerd over de rol van een AMP provocatietest bij astma.



Figuur. Verschil in werkingsmechanisme tussen histamine/methacholine en AMP.

Een direct verband tussen de PC_{20} AMP en de ernst van luchtwegontsteking wordt gevonden in **hoofdstuk 3**. Dit hoofdstuk beschrijft de correlatie tussen de PC_{20} AMP en PC_{20} methacholine aan de ene kant en de ernst van luchtwegontsteking in opgewekt sputum aan de andere kant bij 120 astmapatiënten. Dit onderzoek toont aan dat de PC_{20} AMP veel beter gecorreleerd is met luchtwegontsteking in astma dan de PC_{20} methacholine.

Studies hebben aangetoond dat de PC_{20} AMP meer verbetert na behandeling met ontstekingsremmers (corticosteroïden) dan de PC_{20} methacholine. Het is echter niet bekend

of deze verbetering inderdaad een afspiegeling is van een sterkere afname van inflammatie of dat de afname van de PC_{20}-waarde een rekenkundig gevolg is van het feit dat er meer concentratiestappen nodig zijn bij een AMP provocatietest dan bij een methacholine provocatietest. Daarom hebben wij in **hoofdstuk 4** de correlatie tussen de corticosteroïd-geïnduceerde verbetering in PC_{20} AMP en de vermindering van inflammatie onderzocht. Hiervoor werd de behandeling met corticosteroïden eerst stapsgewijs afgebouwd en uiteindelijk gestopt bij 120 astmapatiënten. Vervolgens werd begonnen met een behandeling van twee weken met een hoge dosering corticosteroïden. Voor en na deze behandeling van twee weken werden zowel een provocatietest met AMP en met methacholine verricht als ook sputum opgewekt. De resultaten van dit onderzoek tonen aan dat de afname van de PC_{20} AMP waarde daadwerkelijk beter gecorreleerd is met een afname van inflammatie dan de afname van de PC_{20} methacholine waarde. De resultaten van het onderzoek in hoofdstuk 3 en 4 samen zijn een goede aanwijzing dat de PC_{20} AMP gebruikt kan worden als een methode om luchtweg inflammatie te meten en om het effect van corticosteroïden te monitoren.

Bij een AMP provocatietest inhaleren patiënten AMP via een vernevelaar. AMP wordt ook in het lichaam zelf gevormd tijdens het omzetten van hoog energetische fosfaatverbindingen (Adenosine Difosfaat (ADP) en Adenosine Trifosfaat (ATP)) bij het genereren van energie. Normaliter wordt AMP uiteindelijk weer omgezet naar ADP en ATP als onderdeel van de energiecyclus. Echter, in een situatie van grote energiebehoefte of zuurstoftekort wordt AMP omgezet naar adenosine. Het is dan ook niet verrassend dat de concentratie adenosine in de luchtwegen van astmatici verhoogd is en verder toeneemt na allergeen provocatie. Er zijn aanwijzingen in de literatuur dat adenosine, eenmaal geproduceerd in de luchtwegen, zelf een rol speelt bij het onderhouden van de luchtwegontsteking. Zo is het bijvoorbeeld aangetoond dat muizen die het enzym 'adenosine deaminase' missen, en daardoor niet in staat zijn adenosine af te breken, luchtwegontsteking en kenmerken van astma ontwikkelen. Met het onderzoek beschreven in **hoofdstuk 5**, hebben wij onderzocht of een provocatietest met AMP tot een toename van luchtwegontsteking leidt. Wij vonden dat het aantal ontstekingscellen in opgewekt sputum toeneemt na een provocatietest met AMP. Ondanks deze toename van luchtwegontsteking lijkt het niet waarschijnlijk dat een provocatietest met AMP schadelijk is voor de luchtwegen, omdat in een eerder onderzoek is aangetoond dat de longfunctie en luchtwegreactiviteit stabiel blijven tot ten minste 24 uur na een provocatietest met AMP.

Tot nu toe zijn er vier verschillende receptoren voor adenosine beschreven, namelijk adenosine A_1, A_{2A}, A_{2B}, en A_3 receptoren. De A_{2B} receptor bevindt zich op de mestcel en activatie van deze receptor leidt tot het vrijkomen van ontstekingsmediatoren. Adenosine A_1, A_{2A}, en A_3 receptoren bevinden zich op neutrofielen, eosinofielen en macrofagen. In de literatuur wordt melding gemaakt dat activatie van deze receptoren soms aanleiding geeft tot ontsteking, maar soms ook de ontsteking remt. Activatie van adenosine A_1 receptoren leidt tot een toename van neutrofielen (een ontstekingscel in bloed die ook in longweefsel voorkomt) en een verhoogde adherentie van neutrofielen aan endotheelcellen. Activatie van adenosine A_{2A} receptoren daarentegen, leidt tot een vermindering van aantrekken en activatie van neutrofielen. Activatie van adenosine A_3 receptoren tenslotte remt de chemotaxie van eosinofielen. Het is aangetoond bij ratten dat behandeling met medicamenten die de adenosine A_1 receptor remmen (A_1-antagonisten) of de adenosine A_{2A} receptor stimuleren (A_{2A}-agonisten) het astmatische ontstekingsproces afremt. In

hoofdstuk 6 hebben wij onderzocht of behandeling met een A_{2A}-agonist het astmatische ontstekingsproces bij mensen gunstig kan beïnvloeden. Voor dit doeleinde werden patiënten drie keer een week behandeld met ofwel een A_{2A}-agonist, ofwel een nepmedicijn (placebo), ofwel een inhalatiecorticosteroïd. Na steeds één week behandeling werd het effect op de late astmatische reactie en het aantal ontstekingscellen in opgewekt sputum onderzocht. In dit onderzoek was behandeling met een A_{2A}-agonist even weinig effectief als behandeling met placebo. Dit kan betekenen dat òf dit medicament bij mensen niet effectief is òf de dosering die gebruikt is te laag was gekozen.

Laboratoriumstudies hebben aangetoond dat activatie van $ß_2$-receptoren niet alleen leidt tot relaxatie van de spieren rondom de luchtwegen zoals eerder betoogd, maar ook tot een afname in productie van mediatoren door ontstekingscellen. Desalniettemin laten studies in patiënten geen effect zien van regelmatig gebruik van $ß_2$-agonisten op luchtwegontsteking. Een mogelijke verklaring hiervoor is dat de $ß_2$-receptoren die op ontstekingscellen zitten snel ongevoelig raken voor stimulatie door β_2-agonisten. Naast regelmatig gebruik van β_2-agonisten, zijn er aanwijzingen dat β_2-receptoren ook ongevoelig raken door de aanwezigheid van ontstekingsmediatoren. In **hoofdstuk 7** wordt inderdaad aangetoond dat $ß_2$-receptoren ongevoelig worden tijdens de late astmatische reactie. Dit fenomeen kan mogelijk zelf ook weer bijdragen aan een toename van luchtwegontsteking.

CONCLUSIES EN SUGGESTIES VOOR TOEKOMSTIG ONDERZOEK

In de studies beschreven in dit proefschrift hebben wij aangetoond dat de PC_{20} AMP gecorreleerd is met luchtweginflammatie in astma en dat de corticosteroïd geïnduceerde verbetering in PC_{20} AMP gecorreleerd is met vermindering in inflammatie. Dit betekent dat de PC_{20} AMP gebruikt kan worden als een maat voor luchtwegontsteking. Het is zeker de moeite waard om te onderzoeken of het bijstellen van de dosering inhalatiecorticosteroïden op geleide van de PC_{20} AMP een verbetering kan zijn voor de behandeling van astma.

Er zijn belangrijke aanwijzingen dat adenosine een belangrijke rol speelt bij het onderhouden van het ontstekingsproces bij astma. Tot nu toe is nog maar weinig bekend over de precieze functie van de verschillende adenosine receptoren in de luchtwegen. Verder onderzoek is nodig om de rol van adenosine en adenosine receptoren in astma op te helderen. Dit kan gebeuren door het ontwikkelen van medicamenten die de verschillende adenosine receptoren specifiek remmen (adenosine A_1, en A_3 receptor) of stimuleren (A_{2A} receptor) en om te onderzoeken of deze medicamenten een gunstig effect hebben op astma.

DANKWOORD

Hierbij wil ik iedereen bedanken die een bijdrage heeft geleverd aan de totstandkoming van dit proefschrift en een aantal personen in het bijzonder.

Allereerst professor dr. D.S. Postma. Beste Dirkje, het is een groot voorrecht geweest om met je te mogen werken. Jouw kennis van zaken en werktempo zijn indrukwekkend. Maar bovenal wist je mij met je positieve uitstraling en betrokkenheid steeds te inspireren. Ik ben je heel erg dankbaar dat je mij de afgelopen 4 jaar hebt begeleidt in het doen van wetenschappelijk onderzoek en ik heb veel van je geleerd.

Professor dr. H.A.M. Kerstjens, beste Huib. Graag wil ik je bedanken voor jouw altijd positieve instelling. Ik heb een groot respect voor jouw deskundigheid bij het opzetten, uitvoeren en opschrijven van wetenschappelijk onderzoek en ik prijs me gelukkig dat ik hier de afgelopen jaren gebruik van heb mogen maken. Dankzij je lage drempel kon ik altijd bij je binnenlopen, wanneer ik snel advies nodig had en dat heb ik zeer gewaardeerd.

Professor dr. G.H. Koëter, beste Gerard. Jij zorgde er met jouw analyserende en beschouwende blik altijd voor dat de grote lijnen niet verloren gingen. Daarnaast heb ik goede herrineringen aan de gesprekken die niet rechtstreeks mijn promotie betroffen. Tenslotte wil ik je bedanken dat je mij hebt aangenomen voor de opleiding tot longarts.

Professor dr. S.T. Holgate, professor dr. J.W.J. Lammers, professor dr. P.J. Sterk, en professor de Monchy voor hun bereidheid om plaats te nemen in de leescommissie.

De medewerkers van het laboratorium longziekten/allergologie. Als ik soms wat laat met een nieuw project aankwam, kon ik toch altijd bij jullie terecht. Ook waren jullie altijd bereid om sputum of bloed op te werken ook wanneer een afspraak een keer niet goed was doorgegeven of als er gewerkt moest worden op een vrije dag. Heel erg bedankt daarvoor. Dorothea de Reus wil ik speciaal bedanken voor alle hulp bij het opzetten en uitwerken van nieuwe projecten.

Alle medewerkers van het functiecentrum (longfunctie en allergologie) voor het uitvoeren van de vele longfunctiemetingen en provocatietesten die soms op het laatste moment moesten gebeuren.

Alle leden van het GRIAC voor de stimulerende discussies en hun waardevolle commentaar tijden de research besprekingen. Ik heb het als een voorrecht beschouwd om deel uit te maken van zo'n grote groep waar zo veel disciplines vertegenwoordigd waren.

Evelyn en Trudy voor de vele klusjes die jullie altijd vlot opknapten.

Erwin, Jenny, Gerben, Rolf, en Anne van het bedrijfsbureau interne geneeskunde voor de ondersteuning bij dit onderzoek.

Ilse en Josefien voor hun enorme hulp en inzet bij het FFA onderzoek. Dit heeft het voor mij mogelijk gemaakt om het proefschrift voor het grootste deel af te ronden voor de start van mijn opleiding tot longarts.

Tot slot mijn kamergenoten en directe collega's, Jeroen, Jos, Floris, Maurits, Hester, Margot, Henk, Erik, Hajo, Toby, Marlies, Gerard, Brigitte, en Jaap voor de gezellige sfeer, de adviezen, de vele discussies, en altijd hulp wanneer dat nodig was.